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BIOQUÍMICA TOXICOLÓGICA

PARÂMETROS TOXICOLÓGICOS EM CARPAS (*Cyprinus carpio*) EXPOSTOS A FORMULAÇÕES COMERCIAIS DE DIFERENTES HERBICIDAS EM CONDIÇÕES DE LAVOURA DE ARROZ E EM LABORATÓRIO

DISSERTAÇÃO DE MESTRADO

Bibiana Silveira Moraes

Santa Maria - RS, Brasil

2008

PARÂMETROS TOXICOLÓGICOS EM CARPAS (*Cyprinus carpio*) EXPOSTOS A FORMULAÇÕES COMERCIAIS DE DIFERENTES HERBICIDAS EM CONDIÇÕES DE LAVOURA DE ARROZ E EM LABORATÓRIO

por

Bibiana Silveira Moraes

Dissertação apresentada ao 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 BIOQUÍMICA TOXICOLÓGICA**

Orientador: Prof. Dr. Vania Lucia Loro

Santa Maria – RS, Brasil

2008

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
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A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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elaborada por

Bibiana Silveira Moraes

como requisito parcial para a obtenção do grau de
Mestre em Bioquímica Toxicológica

COMISSÃO EXAMINADORA

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Profª. Drª. Vania Lucia Loro
(Orientador – Presidente)

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Prof. Drª. Maria Ester Pereira (UFSM)

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Prof. Dr. João Radünz Neto (UFSM)

Santa Maria, 20 de agosto de 2008.

Dedico este trabalho aos meus pais, pois sei
que através desta conquista eles também
estão se realizando,
não só profissionalmente mas
também pessoalmente.
Obrigada pelas horas e horas de conselhos, opiniões,
enfim todo o incentivo de sempre....
com certeza este trabalho é o resultado
do amor, do carinho, da dedicação e
da experiência de vida que me foi passado.
Amo muito vocês!

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pelo imenso apoio em todas as horas,
nas conquistas alcançadas, mas principalmente
nos momentos de angústias, de dúvidas,
e de tomada de decisões importantes.
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Te amo!!

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Meus agradecimentos aos amigos e pessoas que de uma forma ou de outra auxiliaram para a realização deste trabalho e que com pesar não mencionei nesses agradecimentos seletivos;

"Toda a busca,
exige uma meta bem definida,
para se obter o sucesso."

RESUMO

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

PARÂMETROS TOXICOLÓGICOS EM CARPAS (*Cyprinus carpio*) EXPOSTOS A FORMULAÇÕES COMERCIAIS DE DIFERENTES HERBICIDAS EM CONDIÇÕES DE LAVOURA DE ARROZ E EM LABORATÓRIO

AUTORA: BIBIANA SILVEIRA MORAES
ORIENTADOR: VANIA LUCIA LORO
Data e Local da Defesa: Santa Maria, 20 de agosto de 2008.

Os pesticidas podem afetar parâmetros toxicológicos e ainda causar estresse oxidativo em peixes. No Brasil existem poucos estudos relacionando toxicidade de pesticidas com peixes. Por esta razão foram conduzidos dois experimentos com objetivo de avaliar possíveis efeitos dos herbicidas sobre alguns parâmetros toxicológicos em carpas (*Cyprinus carpio*). Os peixes foram expostos a dois herbicidas comerciais, um composto pela mistura formulada de imazetapir e imazapic e o segundo composto por clomazone. Os períodos de exposição foram de sete dias em laboratório e por sete, 30 ou 90 dias em condição de lavoura de arroz. Após os períodos experimentais, nos tecidos hepático, cerebral e muscular foram avaliados parâmetros metabólicos e toxicológicos. Os parâmetros enzimáticos analisados foram a atividade da acetilcolinesterase (AChE), catalase (CAT) e glutationa S-transferase (GST) em diferentes tecidos desta espécie. Além disso, analisaram-se parâmetros de estresse oxidativo, como a carbonilação de proteínas e níveis de TBARS no tecido hepático. Os parâmetros metabólicos analisados foram glicose, glicogênio, lactato, proteína, amônia e aminoácidos em fígado e músculo de carpas. Ademais, foram feitas avaliações metabólicas (glicose, lactato e proteína) em plasma de carpas. Os resultados mostraram que a atividade da AChE foi aumentada em cérebro de carpas, após sete dias de exposição a formulação comercial contendo os herbicidas imazetapir e imazapic, em condições de campo e laboratório. Neste mesmo período de exposição, o tecido muscular mostrou redução da atividade da AChE após exposição as duas formulações comerciais em laboratório. Aos 30 dias em campo, a atividade da enzima AChE foi reduzida em cérebro e aumentada em músculo de carpas expostas a formulação contendo a mistura dos herbicidas imazetapir e imazapic. Os peixes expostos ao herbicida clomazone não mostraram alteração na atividade desta enzima, neste período. Já aos 90 dias de exposição, somente os peixes expostos ao herbicida clomazone, mostraram uma redução na atividade da AChE muscular. A enzima antioxidante catalase mostrou sua atividade aumentada no tecido hepático após sete dias de exposição em ambas as condições experimentais e às duas formulações comerciais testadas. Aos 30 dias de exposição, a enzima não foi alterada e após 90 dias de exposição a sua atividade foi reduzida em fígado de carpas expostas ao herbicida clomazone. A enzima glutationa S-transferase só foi alterada após 30 e 90

dias de exposição em campo. Sua atividade foi reduzida no tecido hepático após exposição às formulações comerciais dos herbicidas testados. Os níveis de TBARS foram aumentados em praticamente todos os períodos, condições de exposição e tecidos considerados, com exceção do tecido cerebral que aos 30 dias de exposição, mostrou os níveis de TBARS diminuídos. Os níveis de proteína carbonil foram diminuídos em fígado de carpas expostas por sete dias ao herbicida clomazone em laboratório. Por outro lado, aos 30 e 90 dias de exposição os níveis de proteína carbonil foram aumentados no fígado de peixes expostos as duas formulações comerciais. Após exposição aos dois herbicidas comerciais, as carpas demonstraram desordens metabólicas. De uma maneira geral, os níveis de proteína e aminoácidos foram diminuídos e o conteúdo de glicogênio aumentado em fígado e músculo de carpas expostas aos dois herbicidas comerciais e tempos testados. Porém, os níveis de amônia, lactato e glicose mostraram alterações variáveis de acordo com o tecido considerado, com o tempo de exposição e com o herbicida testado. Estes resultados indicam que os parâmetros medidos podem ser bons indicadores da contaminação destes herbicidas comerciais em *Cyprinus carpio.*

Palavras-chave: carpa (*Cyprinus carpio*), herbicidas, estresse oxidativo, AChE, parâmetros metabólicos.

ABSTRACT

Master Dissertation
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

TOXICOLOGICAL PARAMETERS IN CARP (*Cyprinus carpio*) EXPOSED TO COMMERCIAL FORMULATIONS OF DIFFERENT HERBICIDES IN RICE FIELD AND UNDER LABORATORY CONDITIONS

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ADVISOR: VANIA LUCIA LORO
Data and Place of the defense: August, 20th, 2008, Santa Maria

Pesticides can affect toxicological parameters and cause oxidative stress in fish. In Brazil there are few studies linking pesticide toxicity with fish. For this reason, two experiments were conducted to evaluate possible effects of herbicides on some toxicological parameters in carp (*Cyprinus carpio*). The fish were exposed to two commercial herbicides, one composed of a mixture formulated imazetapir and imazapic and the second composed of clomazone. The periods of exposure were seven days in the laboratory and seven, 30 or 90 days on rice field condition. After the experimental period, metabolic and toxicological parameters in the liver, brain and muscle tissues were evaluated. The enzymatic parameters analyzed in different tissues of this species were acetylcholinesterase activity (AChE), catalase (CAT) and glutathione S-transferase (GST). Also, oxidative stress parameters, such as carbonyl protein and TBARS levels in hepatic tissue, were analyzed. The metabolic parameters analyzed in liver and muscle of carp were glucose, glycogen, lactate, protein, amino acids and ammonia. Moreover, metabolic assessments were made (glucose, lactate and protein) in carp plasma. The results showed that AChE activity was increased in carp brain after seven days of exposure to a commercial formulation containing imazethapyr and imazapic herbicides both under field and laboratory conditions. In the same period of exposure, muscle tissue showed reduced activity of AChE after exposure to all herbicides (imazetapir and imazapic and clomazone) under laboratory conditions. At 30 days, in the field, AChE activity was reduced in brain and enhanced in muscle of carps exposed to imazethapyr and imazapic. Fish exposed to clomazone herbicide did not show any change in the activity of this enzyme in this period. On the other hand, at 90 days of exposure, only the fish exposed to the herbicide clomazone, showed a reduction in muscle AChE activity. The antioxidant enzyme catalase showed increased activity in liver tissue after seven days of exposure under both experimental conditions. At 30 days of exposure, catalase activity showed no further changes and after 90 days of exposure activity was reduced in liver of carp exposed to clomazone herbicide. The enzyme glutathione S-transferase was altered only after 30 and 90 days of exposure in the field. GST activity was reduced in liver tissue after exposure to both herbicides tested. TBARS levels were increased in almost all periods, terms of exposure and tissues considered, with the exception of brain tissue, which presented a decrease in TBARS levels at 30 days of exposure. Protein carbonyl was reduced in liver of carp exposed to clomazone herbicide after seven days under laboratory conditions. Moreover, at 30 and 90 days of exposure, the protein carbonyl levels were increased in the liver of fish exposed to both herbicides. After exposure to both herbicides, the carp showed metabolic disorders. In general, protein and amino acid levels were

reduced and the amount of glycogen was enhanced in liver and muscle of carp exposed to both herbicides at all times tested. However, ammonia, lactate and glucose levels were increased and reduced according to the tissue considered, time of exposure and herbicide tested. These results indicate that the parameters measured may be good indicators of contamination of these commercial herbicides in *Cyprinus carpio.*

Key words: carp (*Cyprinus carpio*), herbicides, oxidative stress, AChE, metabolic parameters.

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LISTA DE ABREVIATURAS

ACh: acetilcolina

AChE: acetilcolinesterase

AHAS: acetohidróxiácido sintase

ALS: acetolactato sintase

BChE: butirilcolinesterase

CAT: catalase

DNPH: 2,4-dinitrofenilhidrazina

DTNB: ácido 5,5´-ditio-bis-2-nitrobenzóico

EROs: espécies reativas de oxigênio

GPx: glutationa peroxidase

GST: glutationa S-transferase

H_2O_2: peróxido de hidrogênio

LPO: peroxidação lipídica

MDA: malondialdeído

TBA: ácido 2-tiobarbitúrico

TBARS: substâncias reativas ao ácido tiobarbitúrico

SDS: lauril sulfato de sódio ou duodecil sulfato de sódio

SNC: sistema nervoso central

SNP: sistema nervoso periférico

SOD: superóxido dismutase

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APRESENTAÇÃO

Esta dissertação está descrita da seguinte forma: primeiramente são apresentados a **INTRODUÇÃO, os OBJETIVOS e a REVISÃO BIBLIOGRÁFICA**.

A seguir, os **RESULTADOS** são apresentados na forma de **MANUSCRITOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos e representam a integra deste trabalho.

No final da dissertação encontram-se os itens **DISCUSSÃO e CONCLUSÕES**, nos quais há interpretações e comentários gerais sobre os manuscritos contidos neste estudo.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA, DISCUSSÃO e CONCLUSÕES** desta dissertação.

1 INTRODUÇÃO

Nos últimos anos há um aumento na preocupação com a contaminação ambiental, principalmente no que se refere à contaminação das águas. Um dos fatores que contribui para esta contaminação é o uso freqüente de pesticidas na agricultura (SANCHO *et al*., 2000; ORUÇ *et al*., 2004). Pesticidas são todas as substâncias ou misturas que tem como objetivos impedir, destruir, repelir ou mitigar qualquer praga. É um termo utilizado que agrupa herbicidas (controlam plantas daninhas), inseticidas (usado no controle de insetos), fungicidas (controlam fungos causadores de doenças), nematicidas (controlam nematóides) e acaricidas (usados no controle de ácaros), todos muito utilizados na agricultura.

Os herbicidas imazetapir e imazapic pertencem ao grupo das imidazolinonas e são registrados no Brasil para o controle de plantas daninhas em diferentes culturas (RODRIGUES & ALMEIDA, 2005). Estes herbicidas atuam sobre enzimas que estão presentes apenas nas espécies vegetais, como é o caso destes herbicidas inibidores da enzima acetolactato sintase (ALS). O clomazone é um herbicida que pertence ao grupo das isoxazolidinonas sendo registrado no Brasil para diversas culturas no controle de plantas daninhas em pré-emergência (RODRIGUES & ALMEIDA, 2005). Os resíduos de clomazone podem ser encontrados em cerca de 90% de águas coletadas de rios próximos às plantações de arroz (ZANELLA *et al*., 2002).

A ocorrência de poluentes em ambientes aquáticos pode afetar a saúde e a sobrevivência dos peixes (DEZFULI *et al*., 2003). As águas que saem das lavouras, por exemplo, podem afetar diretamente ou indiretamente a vida de muitos organismos não-alvo, como os peixes (ORUÇ & ÜNER, 1999; BRETAUD *et al*., 2000). Recentemente, um grande número de estudos tem mostrado efeitos de pesticidas sobre os organismos aquáticos, principalmente estudos que utilizam doses sub-letais dos produtos (CRESTANI *et al*., 2006; GLUSCZAK *et al*., 2007; MORAES *et al*., 2007; FONSECA *et al*., 2008). Os processos de transporte e de impacto sobre organismos não-alvo são coordenados pelas taxas de degradação e pela biodisponbilidade desses pesticidas no solo ou na água. A biodisponibilidade do pesticida depende de suas características físico-químicas, bem como das condições edafoclimáticas onde ele se encontra. Os efeitos sobre os peixes variam de acordo com a espécie, estágio de crescimento, com o produto, com a dose, concentração

do produto, tempo de exposição (CRESTANI *et al*., 2007; GLUSCZAK *et al*., 2006; MORAES *et al*., 2007; FONSECA *et al*., 2008; CATTANEO *et al*., 2008).

No Brasil, os estudos de toxicidade de pesticidas em peixes são bastante escassos, principalmente quando associados às culturas agrícolas. Então a partir do comportamento dos pesticidas no ambiente e a possibilidade de contaminação da água, torna-se de interesse analisar seus possíveis efeitos tóxicos em peixes de interesse comercial como as carpas. A carpa húngara (*Cyprinus carpio*) é originária da Europa Oriental e da Ásia Ocidental. Atualmente seu cultivo ocorre em todos os continentes, devido a sua rusticidade, resistindo a diferentes temperaturas e facilidade de criação (QUEROL *et al*., 2005).

Contaminantes ambientais, entre eles os pesticidas, podem provocar um aumento na produção de espécies reativas em diversos organismos aquáticos, como os peixes, ocasionando assim uma situação de estresse oxidativo (AHMAD *et al*., 2000; ÜNER *et al*., 2005). Quando ocorre um grande aumento das espécies reativas de oxigênio (EROs) intracelular, a capacidade de defesa antioxidante é prejudicada, ocorre um desequilíbrio entre pró-oxidantes e antioxidantes, ocasionando o chamado estresse oxidativo (ÜNER *et al*., 2006). O sistema antioxidante pode ser enzimático e não enzimático. As enzimas superóxido dismutase, catalase e glutationa-S-transferase são responsáveis pela proteção celular contra as EROs. Estudos mostram que quando as atividades destas enzimas se encontram alteradas em peixes, elas podem ser usadas como indicadores de exposição aos poluentes aquáticos (AHMAD *et al*., 2000; LI *et al*., 2003, MORAES *et al*., 2007). Em uma situação de estresse oxidativo pode ocorrer ainda a peroxidação lipídica que é observada pelo aumento de malondialdeído (ALMROTH *et al*., 2005) e a carbonilação de proteínas que indica possíveis danos causados as proteínas (PARVEZ & RAISUDDIN, 2005).

Além dos parâmetros de estresse oxidativo, a medida da atividade da enzima acetilcolinesterase (AChE) é utilizada para avaliar a toxicidade de pesticidas em peixes. Esta enzima tem sido utilizada por diferentes autores como um marcador para diagnosticar a exposição a compostos como carbamatos e organofosforados (CHUIKO, 2000; SANCHO *et al*., 2000; FERNÁNDEZ-VEGA *et al*., 2002). Porém, em outros estudos verificou-se que diferentes classes de pesticidas também causam alterações na atividade da AChE de cérebro ou músculo de peixes (DUTTA &

ARENDS, 2003; MIRON *et al*., 2005; GLUSCZAK *et al*., 2006; MORAES *et al*., 2007).

A presença de pesticidas na água também pode ocasionar alterações fisiológicas em peixes resultando na adaptação do organismo ao contaminante ou na indução de efeito negativo sobre a sobrevivência e condição de saúde do peixe (BEGUM, 2004). Por isso, possíveis alterações no metabolismo causadas por pesticidas podem ser avaliadas através de parâmetros metabólicos em diferentes tecidos de peixe (BEGUM & VIJAYARAGHAVAN, 1995; SANCHO *et al*., 1998; BEGUM, 2004; CRESTANI *et al*., 2006).

Dado o exposto, considerando a importância de conhecer possíveis efeitos adversos das referidas formulações comerciais em carpas, pretende-se verificar se a exposição em condições de laboratório e a exposição em campo no sistema de arroz irrigado alteram parâmetros de toxicidade destes organismos.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito de algumas formulações comerciais dos herbicidas imazetapir, imazapic e clomazone sobre parâmetros enzimáticos, de estresse oxidativo e metabólicos, em juvenis de carpa húngara em sistema de arroz irrigado e em laboratório.

2.2 Objetivos específicos

- Investigar os efeitos dos herbicidas sobre a atividade da enzima acetilcolinesterase (AChE) cerebral e muscular em carpas.

- Determinar a atividade de enzimas antioxidantes (catalase e glutationa-S-transferase) de fígado em peixes expostos aos herbicidas.

- Investigar o possível estresse oxidativo, evidenciado por peroxidação lipídica, através da determinação de substâncias reativas ao ácido tiobarbitúrico (TBARS) no tecido hepático, cerebral e muscular e da carbonilação de proteínas em fígado.

- Verificar se ocorre alterações nos parâmetros metabólicos (proteína, lactato, glicose, glicogênio, aminoácidos e amônia total) em fígado, músculo e plasma dos peixes.

- Relacionar os resultados obtidos em laboratórios com os obtidos no experimento de campo.

3 REVISÃO BIBLIOGRÁFICA

3.1 Herbicidas

3.1.1 Imazetapir e imazapic

No Brasil, o herbicida Only® foi desenvolvido para uso exclusivo no sistema de produção Clearfield-Arroz. Este herbicida é uma mistura de dois herbicidas: imazetapir e imazapic (75 g/L e 25 g/L, respectivamente) e o restante (920 g/L) são ingredientes inertes. Na planta estes herbicidas atuam como inibidores da enzima acetolactato sintase (ALS) ou acetohidróxiácido sintase (AHAS) pertencentes ao grupo químico das imidazolinonas. A enzima ALS é inibida poucas horas após a aplicação do herrbicida sobre a cultura, causando redução do crescimento da planta e injúrias como a clorose meristemática (SENSEMAN, 2007). Apresentam como mecanismo de ação a inibição da síntese dos aminoácidos alifáticos de cadeia lateral: valina, leucina e isoleucina (CHRISTOFFOLETI *et al.*, 2004).

O imazetapir, {2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid} (Figura 1) é um herbicida com características de ácido orgânico fraco (pKa 3,9) que tem solubilidade em água de 1400 mg L^{-1} (pH 7; 25ºC) e pressão de vapor <0.013 mPa (60ºC). A meia-vida no solo desse herbicida varia de 60 a 90 dias e suas perdas por volatilização são insignificantes (SENSEMAN, 2007).

O imazapic, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-methyl-3pyridinecarboxylic acid (Figura 2) consiste também num herbicida com características de ácido fraco (pKa 3,9), com solubilidade em água de 2200 mg L^{-1} (25ºC). A meia-vida do herbicida no solo é de 120 dias e, assim como para o imazetapir, suas perdas por volatilização são insignificantes (SENSEMAN, 2007). Os possíveis efeitos destes herbicidas em peixes ainda são desconhecidos.

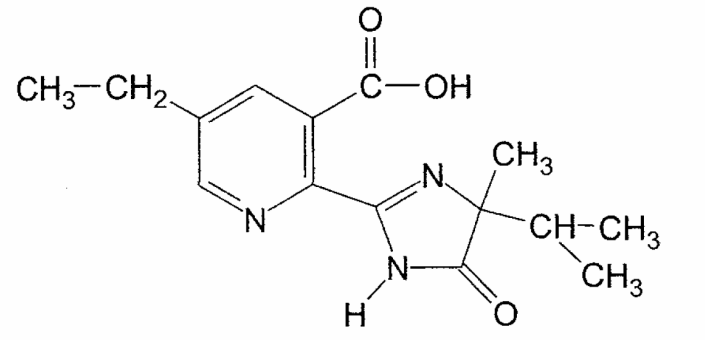

Figura 1: Estrutura química do herbicida imazetapir (adaptado de Senseman, 2007).

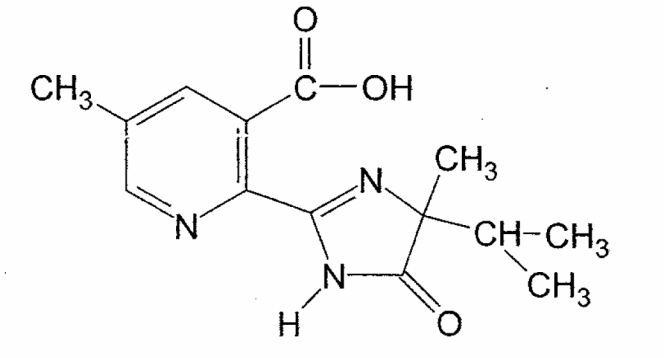

Figura 2: Estrutura química do herbicida imazapic (adaptado de Senseman, 2007).

3.1.2 Clomazone

O herbicida clomazone 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone (Figura 3) é registrado no Brasil para o controle de plantas daninhas em pré-emergência em diversas culturas, dentre elas o arroz irrigado. Pertence ao grupo químico das isoxazolidinonas, é absorvido pelas raízes e move-se no xilema até as folhas das plantas (RODRIGUES & ALMEIDA, 2005). Os herbicidas deste grupo causam inibição da síntese de carotenóides. O caroteno é um pigmento das plantas responsável, dentre outras funções, pela proteção da clorofila da foto-oxidação, portanto as plantas suscetíveis apresentam o albinismo como o principal sintoma, causando uma aparência descorada (CHRISTOFFOLETI *et al.*, 2004). O clomazone tem solubilidade em água de 1100 mg L^{-1} (25ºC) e pressão de vapor de

19.2 mPa (25ºC). A meia-vida do clomazone no solo é de 24 dias, mas esse período pode variar com o tipo do solo e as circunstâncias ambientais (SENSEMAN, 2007).

Estudos recentes têm avaliado a exposição de peixes ao herbicida clomazone (MORAES *et al*., 2007; CRESTANI *et al*., 2007). Miron *et al*. (2005) concluíram que após a exposição de jundiás (*R. quelen*) a altas concentrações (5, 10 ou 20 mg/L) do herbicida clomazone, a atividade da enzima AChE foi inibida em até 83% no cérebro e 89% no músculo. Em outro estudo, jundiás (*R. quelen*) também expostos ao herbicida clomazone por um período de até 192 h, mostraram alterações nos parâmetros metabólicos. Porém as concentrações utilizadas no estudo foram consideradas seguras para esta espécie, pois no período de recuperação em água livre de herbicida, estes parâmetros retornaram aos valores controle (CRESTANI *et al*., 2006).

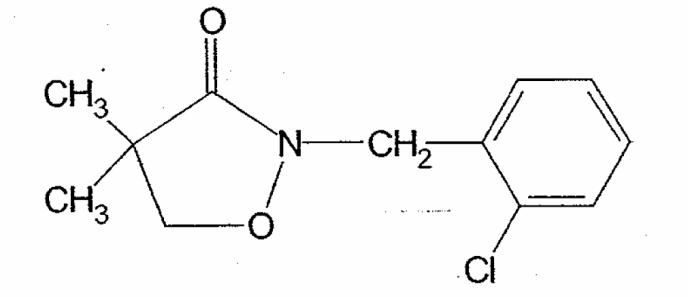

Figura 3: Estrutura química do herbicida clomazone (adaptado de Senseman, 2007).

3.2 Carpa húngara (*Cyprinus carpio* L.1758)

A carpa, peixe da família Cyprinidae, gênero *Cyprinus*, espécie *Cyprinus carpio* L. 1758 (Figura 4) é uma espécie exótica, de origem asiática, criada na China a mais de 2.000 anos (CASTAGNOLLI & CYRINO, 1986). Em 1887, veio para a América, sendo aclimatada nos Estados Unidos. No Brasil, onde se adaptou com grande facilidade, foi introduzida no Estado de São Paulo, em 1904; entretanto, as criações intensivas só tiveram início na década de 30 (GALLI & TORLONI, 1989). No Brasil, muitas pisciculturas utilizam a carpa Húngara, *Cyprinus carpio*, sendo uma das primeiras espécies a serem cultivadas em Aqüicultura. Atualmente seu cultivo

ocorre em todos os continentes, devido a sua rusticidade, por resistirem a grandes diferenças de temperatura e por sua facilidade de criação (QUEROL *et al*., 2005).

Segundo Moreira *et al* (2001), as carpas, pela sua capacidade de resistir a uma ampla faixa de temperatura, são hoje animais cosmopolitas, sendo que seu crescimento ótimo dá-se com temperatura média de 28ºC, só tem seu crescimento afetado abaixo de 15ºC. Não se reproduz quando a temperatura cai abaixo de 20ºC e cessa a ingestão de alimentos quando a temperatura da água é inferior a 4ºC (CASTAGNOLLI & CYRINO, 1986). Resiste bem às quedas do teor de oxigênio dissolvido, suportando até 3,2 mg/L. Porém, pára de se alimentar com nível de 2,5 mg/L e pode morrer com 0,8 mg/L (MENEZES & YANCEY, 1984; GALLI & TORLONI, 1989). É uma espécie onívora que se alimenta de invertebrados, plantas, algas, consome larvas de insetos e crustáceos, podendo alimentar-se também de pequenos peixes (QUEROL *et al*, 2005).

Por ser a carpa cosmopolita, surgiram várias raças, segundo a região e o método de criação. As raças diferem principalmente por características ligadas ao formato, às escamas e ao tamanho da cabeça em relação ao corpo. A carpa Húngara possui um pequeno número de escamas, sendo estas maiores que as da carpa comum, dispostas em três fileiras, na região dorsal, sobre a linha lateral e na região ventral. Apresenta crescimento precoce, uma alta relação entre altura e comprimento do corpo podendo atingir mais de 20 kg (MOREIRA *et al*., 2001).

Figura 4: Exemplar de carpa húngara (*Cyprinus carpio*) juvenil.

3.3 Enzima Acetilcolinesterase

As colinesterases estão amplamente distribuídas entre os animais, desempenhando papéis importantes na neurotransmissão colinérgica central e periférica, além de funções como a hidrólise dos ésteres de colina e a detoxificação de xenobióticos (BRETAUD *et al*., 2000; ROEX *et al*., 2003). Existem duas famílias de colinesterases: a acetilcolinesterase (AChE; EC 3.1.1.7), que hidrolisa preferencialmente ésteres com grupamento acetil (como a acetilcolina) e a butirilcolinesterase (BChE; EC 3.1.1.8) que prefere hidrolisar outros tipos de ésteres como a butirilcolina (NIGG & KNAAK, 2000).

A enzima acetilcolinesterase está presente no sistema nervoso central (SNC), sistema nervoso periférico (SNP) e também nos glóbulos vermelhos do sangue. Essa enzima é responsável por catalisar a degradação da acetilcolina em colina e acetato na fenda sináptica. A atividade da AChE pode variar de acordo com espécie de peixe. Chuiko (2000) mostrou que o nível da atividade específica da acetilcolinesterase em cérebro de peixes que representavam a família Cyprinidae foi maior que os das famílias Percidae e Esocidae. A medida da atividade da enzima acetilcolinesterase é muito utilizada para avaliar a toxicidade de contaminantes ambientais em peixes. Esta enzima tem sido utilizada por diferentes autores como um marcador para diagnosticar a exposição a compostos como carbamatos e organofosforados (SANCHO *et al*., 2000). Porém, em outros estudos, verificou-se que diferentes classes de agrotóxicos também causaram alterações na atividade da AChE em cérebro ou músculo de peixes (MIRON *et al*., 2005; CRESTANI *et al*., 2006; MORAES *et al*., 2007).

O efeito mais comum da exposição aos agrotóxicos é a inibição da atividade da acetilcolinesterase em peixes. A inibição de sua atividade resulta em estimulação excessiva dos nervos colinérgicos, que pode resultar em tremores, nado errático, convulsões e até mesmo a morte (FERNÁNDEZ-VEGA *et al*., 2002). DUTTA & ARENDS (2003), avaliando o inseticida endosulfan, encontraram uma significativa inibição da atividade da AChE em cérebro de *Lepomis macrochirus* quando expostos por até uma semana a concentração de 1,2 µg/L do produto. GLUSCZAK *et al* (2006) em outro estudo, mostraram que a atividade da AChE foi inibida em cérebro de piavas (*Leporinus obtusidens*) quando estes peixes foram expostos a diferentes

concentrações da formulação comercial do herbicida glifosato por um período de 96hs. Em um estudo realizado no campo (em lavoura de arroz irrigado) por um período de 30 dias de exposição, foi observada a inibição da enzima em cérebro de piavas após serem expostas às formulações comerciais dos herbicidas clomazone e quinclorac (MORAES *et al*., 2007;).

Estudos recentes têm demonstrado que a atividade da AChE em tecidos de peixes pode variar de acordo com a espécie, com o tempo de exposição, com a condição experimental em que o peixe se encontra e com o tipo de tóxico a qual o peixe é exposto (MIRON *et al*., 2005; CRESTANI *et al*., 2006; MORAES *et al*., 2007; GLUSCZAK *et al*., 2007; FONSECA *et al*., 2008).

3.4 Estresse oxidativo

As reações de oxidação são essenciais no metabolismo normal dos organismos aeróbicos, principalmente porque o elemento oxigênio atua como aceptor do último elétron no sistema de fluxo de elétrons, sendo responsável pela geração de energia via fosforilação oxidativa (LUSHCHAK & BAGNYUKOVA, 2006). Em condições fisiológicas normais, a maior fonte de espécies reativas de oxigênio (EROs) é a mitocôndria, pois mais de 90% do O_2 é consumido na cadeia transportadora de elétrons que então produz uma quantidade significativamente grande de EROs (PEY *et al*., 2003). As EROs são produzidas durante a função celular normal de células aeróbicas e além disso, elas podem ser geradas como conseqüência do metabolismo intracelular de compostos exógenos, levando a peroxidação lipídica, oxidação de algumas enzimas e a oxidação e degradação de proteínas (MATÉS, 2000). As EROs incluem o radical anion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\bullet}$), e estas espécies possuem alta reatividade química (BARATA *et al*., 2005).

Atualmente, os organismos aquáticos estão continuamente sendo expostos a diversos contaminantes químicos e por isso efeitos adversos podem surgir como resposta aos diferentes mecanismos de toxicidade destes produtos (BARATA *et a*l., 2005). Uma variedade de poluentes ambientais, dentre eles os pesticidas podem provocar um aumento na produção de EROs em diversos organismos aquáticos, como os peixes, ocasionando assim uma situação de estresse oxidativo (AHMAD *et*

al., 2000; ÜNER *et al*., 2005). O estresse oxidativo é um fenômeno bastante complexo que inicia com a formação de EROs (ALMROTH *et al*., 2008). Pode também ser definido como um desequilíbrio entre pró-oxidantes e antioxidantes, onde a quantidade gerada do primeiro é maior, ocorrendo assim possíveis danos oxidativos (SIES, 1997; ÜNER *et al*., 2006). Diversos autores já evidenciaram estresse oxidativo em peixes (SAYEED *et al*., 2003; BAGNYUKOVA *et al*., 2005; ZHANG *et al*., 2005; PEIXOTO *et al*., 2006; MORAES *et al*., 2007).

As EROs podem modificar todas as macromoléculas celulares, incluindo as proteínas, lipídios e DNA (SIES, 1993) (Figura 5). O seu ataque às proteínas pode ocasionar em clivagem das ligações peptídicas, modificações nos resíduos dos aminoácidos, reações de peptídios com lipídios e com produtos da oxidação dos carboidratos, oxidação dos grupos sulfidrila, formação de proteína carbonil, etc (STADTMAN, 1993 apud LUSHCHAK & BAGNYUKOVA, 2006). A formação de proteína carbonil pode ocorrer como resultado do estresse oxidativo. Recentemente alguns autores têm sugerido que a dosagem de carbonilação de proteínas em peixes pode ser usada como biomarcador complementar de estresse oxidativo (ALMROTH et al., 2005; PARVEZ & RAISUDDIN, 2005).

A peroxidação lipídica (LPO) causa danos importantes no sistema biológico e tem sido muito utilizada como biomarcador de estresse oxidativo em peixes (SAYEED *et al*., 2003). A lipoperoxidação é o resultado da atuação dos radicais livres sobre as membranas biológicas que são ricas em ácidos graxos poliinsaturados (ORUÇ & USTA, 2007). Dentre os lipídios, os ácidos graxos poliinsaturados são os mais sensíveis ao ataque das EROs (LUSHCHAK & BAGNYUKOVA, 2006). O processo de LPO influencia na fluidez da membrana e na integridade das biomoléculas associadas com a membrana (ALMROTH *et al*., 2005). A intensidade da peroxidação lipídica pode ser avaliada de acordo com os níveis dos produtos primários ou ainda com os produtos finais da peroxidação, como por exemplo, o malondialdeído (MDA) que é ensaiado com o ácido tiobarbitúrico e expresso em substâncias reativas ao ácido tiobarbitúrico (TBARS) (LUSHCHAK & BAGNYUKOVA, 2006).

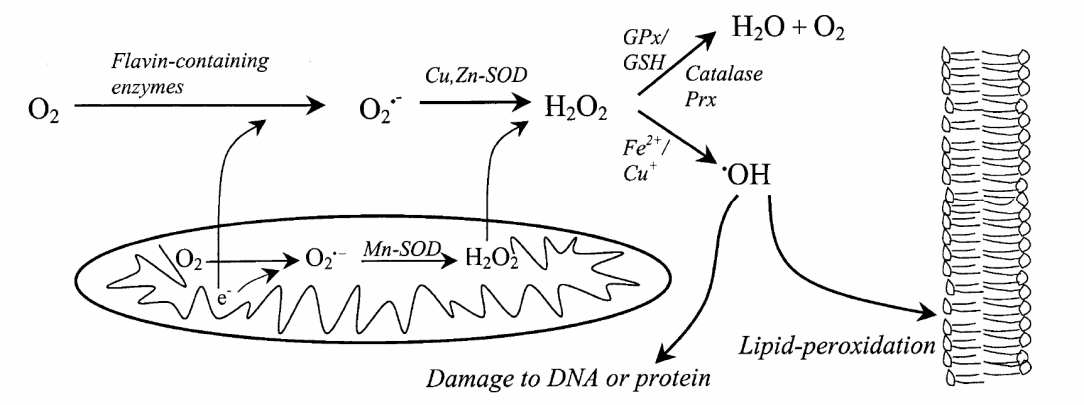

Figura 5: Efeitos das EROs sobre lipídios, proteínas e DNA (adaptado de Nordberg & Arnér, 2001).

Os organismos aeróbicos possuem uma diversidade de defesas antioxidantes para proteger a célula contra os danos ocasionados pela produção de EROs (ZHANG *et al*., 2005). Os peixes também possuem um sistema antioxidante eficaz (TRENZADO *et al*., 2006). Porém, existem poucas informações sobre os mecanismos de defesas que neutralizam os impactos das EROs em peixes (AHMAD *et al*., 2004). O sistema antioxidante pode ser enzimático e não-enzimático. O não-enzimático é composto de metabólitos como a glutationa, ácido ascórbico, tocoferol, etc. As enzimas antioxidantes mais reportadas em organismos aquáticos são a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa S-transferase (GST) (Figura 6). As enzimas CAT e GPx têm papéis complementares na detoxificação do peróxido de hidrogênio, sendo que elas têm diferentes localizações celulares e moléculas alvo (BARATA *et al*., 2005). A CAT é uma das mais importantes enzimas do sistema antioxidante (MATÉS, 2000). Essa enzima se localiza nos peroxissomos e é responsável pela detoxificação do H_2O_2 (ZHANG *et al*., 2005). Quando sua atividade aparece aumentada em determinados tecidos de peixes, isso pode significar uma possível resposta antioxidante do organismo. As glutationa S-transferases são três famílias de enzimas (citosólica, mitocondrial e microssomal), que estão envolvidas na detoxificação de muitos xenobióticos e ainda tem um importante papel na proteção dos tecidos que se encontram em estresse oxidativo (MASELLA *et al*., 2005; ZHANG *et al*., 2005). Este multicomponente enzimático possui um potente efeito protetor contra as EROs (MASELLA *et al*., 2005). A avaliação da GST tem sido bastante reportada em peixes como bioindicador na avaliação de impacto ambiental (AHMAD *et al*., 2000;

PEIXOTO *et al*., 2006; YI *et al*., 2007). Estudos mostram que quando as atividades das enzimas CAT e GST apresentam aumento de suas atividades em tecidos de peixes, elas podem ser usadas como indicadores de exposição aos poluentes aquáticos (AHMAD *et al*., 2000; LI *et al*., 2003, MORAES *et al*., 2007).

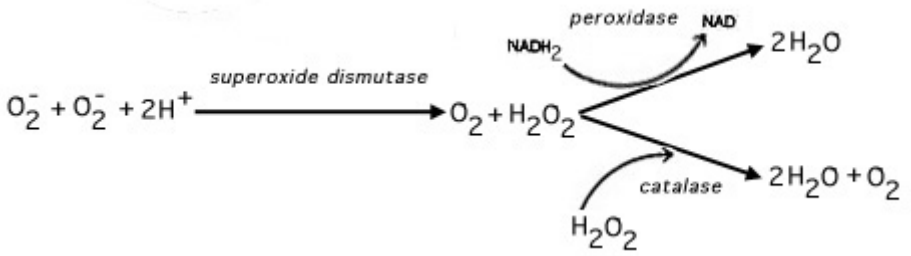

Figura 6: Sistema antioxidante: disponível em http://www.bact.wisc.edu/themicrobialworld/oxygen.jpg

3.5 Intermediários metabólicos

As mudanças que ocorrem na atividade metabólica ou fisiológica de peixes expostos a diferentes poluentes ambientais, dentre eles os agrotóxicos, podem servir como bons indicadores secundários de toxicidade (JYOTHI & NARAYAN, 1999; BEGUM, 2004). O metabolismo de carboidratos que inclui o glicogênio, a glicose e lactato, são frequentemente alterados em tecidos de peixes expostos a agrotóxicos (CRESTANI *et al*., 2006; GLUSCZAK *et al*., 2006, 2007). O metabolismo de proteínas, também é muito importante, uma vez que após exposição à contaminantes ambientais pode ocorrer tanto o catabolismo protéico, quanto a síntese de proteínas e ainda alterações nos níveis de amônia e aminoácidos. Fernández-Vega *et al*. (2002) observaram uma hipoproteinemia em músculo e brânquias de *Anguilla anguilla* expostos a um herbicida da classe dos carbamatos. Ao contrário, Fonseca *et al*. (2008) encontraram níveis de proteínas aumentados em músculo de piavas (*Leporinus obtusidens*) após exposição ao herbicida 2,4-D. Outra resposta fisiológica que pode ocorrer é o aumento dos níveis de amônia em fígado e músculo de peixes expostos a herbicidas (BEGUM, 2004; GLUSCZAK *et al*., 2006, 2007).

O metabolismo do peixe é bastante semelhante ao dos mamíferos, porém com uma grande diferença na quantidade de musculatura branca, que é maior em

peixes. Devido a isso, o metabolismo anaeróbico é bastante utilizado pelos peixes em caso de estresse fisiológico severo, onde a obtenção de energia precisa ser obtida rapidamente. Diversos autores já reportaram alterações nos níveis de lactato hepático, muscular e plasmático (SASTRY & SIDDIQUI, 1982; BEGUM & VIJAYARAGHAVAN, 1999; CRESTANI *et al*., 2006; GLUSCZAK *et al*., 2006). A hiperglicemia é um indicador secundário de estresse, frequentemente reportada em estudos que avaliam o plasma de peixes expostos a agrotóxicos (GIMENO *et al*., 1995; ORUÇ & UNER, 1999; AGUIAR *et al*., 2004; CRESTANI *et al*., 2006; AGRAHARI *et al*., 2007; FONSECA *et al*., 2008).

4 RESULTADOS

4.1 Manuscrito 1

Toxicological responses in different organs of *Cyprinus carpio* after exposure to a commercial herbicide containing imazethapyr and imazapic

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Toxicological responses in different organs of *Cyprinus carpio* after exposure to a commercial herbicide containing imazethapyr and imazapic

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Abstract

Freshwater fish, *Cyprinus carpio* were exposed to a commercial herbicide containing imazethapyr and imazapic (75 + 25 g/L) that correspondent to 0.0075 and 0.0025 mg/L of the formulated herbicide. This is widely used herbicides in the rice field. Fish exposure was done both at laboratory conditions and at field conditions. The laboratory experiment was carried out for seven days and at field experiment exposure was done for 7, 30 and 90 days in a rice field. After exposure, toxicological and metabolic parameters were analyzed. Enzymatic parameters such as acetylcholinesterase (AChE), catalase (CAT) and gluthatione S-transferase (GST) activities were studied in different tissues. Thiobarbituric acid-reactive substances (TBARS), carbonyl protein and metabolic parameters (glycogen, glucose, protein, lactate, ammonia and amino acids) were also studied. After seven days of exposure, brain AChE activity increase in laboratory and field conditions, but in muscle tissue, reduction in activity was observed only in laboratory conditions. At same evaluation period, the oxidative stress parameters only showed changes of TBARS and CAT in laboratory condition. The metabolic parameters also showed changes in both conditions and all exposure period. After 30 days of exposure in rice field condition, the AChE activity in brain decrease and in the muscle the activity was enhanced. After 90 days of exposure in field, the AChE activity was reduced in muscle, but no alteration was observed in brain. The disorders in oxidative stress parameters and metabolism remained in different tissues indicating mainly a protein catabolism. This study pointed out short and long-term effects in two different exposure conditions to environmentally relevant concentrations of rice herbicides containing imazethapyr and imazapic (75 + 25 g/L) on toxicological parameters in tissues and plasma of *Cyprinus carpio.*

KeyWords: *Cyprinus carpio*, protein carbonyl, TBARS, CAT, GST, AChE, metabolism, herbicide.

1. Introduction

Imazethapyr {5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5 dihydroimidazol-1*H*-2-yl)nicotinic acid} and imazapic {2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5methylnicotinic acid}, are imidazolinones herbicides, widely used to control red rice and other weeds in commercial rice. This compound has high solubility in water, 1400 mg/L and 2200 mg/L, respectively (Senseman, 2007). Imazethapyr has a half-life of 53-122 days in aerobic field soil (Mills et al., 1989; Cunan et al., 1992). Several studies have show that the pesticides exposure can to cause biochemical changes in fish (Crestani et al., 2006; Moraes et al., 2007; Cattaneo et al., 2008; Fonseca et al., 2008).

The acetylcholinesterase enzyme activity is frequently decreased after exposure to pesticides. The inhibition of activity could affect growth, survival, feeding, and reproductive behaviors of fish exposed to different pollutants (Dutta & Arends, 2003). However, the effects of enzyme activation are still little known. The AChE activity is a parameter frequently used to environmental monitoring, usually in areas contaminated by pollutants. AChE is an enzyme that catalyses the hydrolysis of acetylcholine into choline and acetate in the synaptic cleft. When occur inhibition in AChE activity, the neurotransmitter acetylcholine (ACh) is not hydrolyzed in nerve synapses and neuromuscular junctions, causing an abnormal amount of ACh at these sites, which leads to overactivation of brain and muscular tissue (Roex et al., 2003).

Furthermore, many pesticides are responsible for causing oxidative stress in aquatic organisms, because these contaminants may induce the formation of reactive oxygen species and alterations in antioxidant system (Monteiro et al., 2006; Üner et al., 2006). Reactive Oxygen Species (ROS) are produced during normal cellular functioning. Fish, as all aerobic organisms, are susceptible to the attack of reactive oxygen species, and their cells have an efficient antioxidant defense system (Trenzado et al., 2006). In a normal condition, a balance exists between formation and elimination of ROS. However, when there is an imbalance caused by the formation of excessive amounts of reactive oxygen species or a deficiency in defense system, occurs the so called cell oxidative stress. The antioxidant system includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). Catalase is one of the most efficient enzymes known, and is

much reported in toxicity studies. The function this enzyme is converting hydrogen peroxide to oxygen and water (Matés 2000; Atli & Canli, 2007). GST is a group of multifunctional enzymes that catalyze the conjugation of GSH with a variety of electrophilic metabolites, which are involved in the detoxification of xenobiotics (Monteiro et al., 2006).

The oxidative stress also can cause lipid peroxidation (LPO). This is a complex process resulting from free radical reactions in biological membranes, which are rich in polyunsaturated fatty acids. LPO in fish is measured as thiobarbituric acid reactive substances (TBARS) has been used as a biomarker in a number of studies and is important in toxicological research (Almroth et al., 2005; Oruç & Usta, 2007). The oxidative stress is to be able of generate hydroxyl radical ($OH^{\bullet}$), that is considered responsible for the formation of carbonyl groups in proteins (Parvez & Raisuddin, 2005). Protein carbonyl has been used as biomarker of exposure in fish, because when to occur carbonylation of proteins, also can occur conformational changes, decreased catalytic activity in enzymes and breakdown of protein by proteases (Almroth et al., 2005).

Not less important are the possible changes in metabolism induced by pesticides exposure. The metabolic parameters are also very used to assess the fish health conditions. Studies have showed that changes in protein and carbohydrate metabolism have occurred in fish that are in stress condition (Glusczak et al., 2007, Fonseca et al., 2008). Carbohydrate and protein metabolism and blood parameters can be used as good indicators to detect the effects of different xenobiotics in fish (Gimeno et al., 1995).

Cyprinus carpio, a native fish of Eastern Europe and Western Asia and widely distributed in Brazil, is an omnivorous species that feeds on invertebrates, plants, algae, consume insects larvae and crustaceans and can feed, also, of small fish (Querol et al, 2005).

The effects of the commercial formulations of herbicides used in rice field condition are scarcely studied in this species of fish. Little is known about the changes in colinergic, antioxidant defense and metabolic systems in response to exposure to herbicide in *Cyprinus carpio*. Thus, the present study aimed to investigate the effects of field concentration commercial formulations of rice herbicide in *C. carpio*, and to determine the possible indicators of fish exposure to this herbicide.

2. Materials and Methods

2.1 Fish

Cyprinus carpio of both genders weighting of 20.0 ± 1.0 g and measuring 11.0 ± 1.0 cm length, were obtained from a fish farm (RS, Brazil). Fish were acclimated to laboratory conditions for 10 days, in tanks (250 L) prior to the experiments. They were kept in continuously aerated water with a static system and with a natural photoperiod (12h light/12h dark).

After acclimation period fish were divided in two groups, a group was transferred to field ponds and the other was laboratory tanks. The study was carried out in two conditions: in a rice field (7, 30 or 90 days) and laboratory (7 days). During all experimental period (90 days) in rice field the average of water parameters were: temperature 24.5 ± 2.0 ^{o}C, pH 6.5 ± 0.2 units, dissolved oxygen 4.21 ± 2.0 mg/L, nonionized ammonia 0.8 ± 0.01 μg/L, nitrite 0.06 ± 0.01 mg/L. In laboratory conditions the average of water parameters were: temperature 22.1 ± 1.0 ^{o}C, pH 7.7 ± 0.2 units, dissolved. Both, in the period of acclimation as in the period of exposure, the fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil).

2.2 Chemicals

The commercial formulation of the herbicide is a mixture of acid equivalent of imazethapyr {5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5 dihydroimidazol-1*H*-2-yl)nicotinic acid} and imazapic {2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5methylnicotinic acid}, Only™ (BASF) that was used in both experiments. Acethylthiocholine (ASCh), 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) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3 Experimental design

2.3.1 Field experimental

Fish were allocated in 2 groups (in triplicate) of 10 animals distributed per tank (30 fish per treatment). One group was the control fish and the other group was the

exposed fish to the herbicide, with concentration corresponding to 0.0 (control) or 0.0075 and 0.0025 mg/L (imazethapyr and imazapic, respectively), for a 7, 30 or 90 days period. The experiment was carried out in the paddy field, with the fishes trapped in submersed cages, measuring 0.30 m (diameter) x 1.05 m (length). Herbicide concentration in water was monitored from the first day until it was not detected. Herbicide was analyzed by High Pressure Liquid Chromatography (HPLC) using the method described by Zanella et al. (2002). After each exposure period (7, 30 and 90 days), a sample of 10 individuals were taken from the tanks and then submitted to blood and tissues (brain, liver and white muscle) collection.

2.3.2 Laboratory experiment

This experiment was carried out in tanks of 30 L capacity (in duplicate) and each tank had 10 fish (20 fish per treatment). The herbicide concentrations were the same used in the field condition. The herbicide was added only in beginning of experiment without water or herbicide replacement. Thus, in this condition was used a filter (with wool acrylic) in each tank to remove feeding waste. These filters were cleaned every day. After the experimental period, the fish were sampled and blood and tissues were collected (brain, liver and white muscle).

2.4 Enzymes assays

2.4.1 Acetylcholinesterase (AChE) activity assay

The AChE (EC 3.1.1.7) activity was measured using the method described by Ellman et al. (1961) and modified by Miron et al. (2005). Brain and muscle tissues (30 mg) were weighted and homogenized in a Potter-Elvejhem glass/Teflon homogenizer with sodium phosphate buffer 50 mM pH=7.2 and Triton X-100 1%. The homogenate was than centrifuged for 10 minutes at 3,000 X *g* at 5°C and the supernatant was used as enzyme source. Aliquots of supernatant (50 and 100 µL) (brain and muscle, respectively) were incubated at 30°C for two minutes with a solution containing 0.1 M sodium phosphate buffer pH 7.5 and 1 mM DTNB. After the incubation period, the reaction was initiated by the addition of ASCh (0.5 mM). The final volume was 2.0 mL. Absorbance was measured by spectrophotometry (Femto Scan spectrophotometer) at 412 nm during 2 min. Enzyme activity was expressed as µmol of ASCh hydrolyzed/min/mg protein.

2.4.2 Catalase activity assay

Catalase (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry (Nelson & Kiesov 1972). Liver tissue were homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.5 (1:20 w/v), centrifuged at 10,000 X *g* for 10 min at 4°C. The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.05 mL homogenate. Change of H_2O_2 absorbance in 60 s was measured by at 240 nm. Catalase activity was calculated and expressed in µmol/min/mg protein.

2.4.3 Glutathione S-tranferase (GST)

GST activity was measured according to Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The formation of S-2, 4-dinitrophenyl glutathione was monitored by the increase in absorbance at 340 nm against blank. The extinction coefficient used for CDNB was 9.6 mM/cm. The activity was expressed as µmol GS-DNB/min/mg protein.

2.5 Carbonyl assay

The liver tissue was homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed by the method described by Yan et al. (1995) with some modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid. After incubation at room temperature for one hour in dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane (99.5%) and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40s and centrifuged at 10,000 X *g* for 15 min. Then, the protein isolated from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1), and suspended in 1 mL of denaturing buffer and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was performed in duplicate and two tubes blank incubated with 2 N HCl without DNPH was included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22,000 M/cm.

2.6 Lipid peroxidation estimation assay

Lipid peroxidation was estimated by a TBARS assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically

measured. Liver, muscle and brain homogenates (100-400 µL) were added TCA 10% and 0.67% thiobarbituric acid were added to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a micro-centrifuge tube and incubated for 15 min at 95 ºC. After cooling, it was centrifuged at 5,000 X *g* for 15 min and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmols MDA/mg protein according to Buege & Aust (1978).

2.7 Protein determination

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

2.8 Metabolic parameters

Liver and muscle glycogen were determined by the method described by Bidinotto et al. (1998) after KOH (6N) and ethanol addition for precipitation of glycogen. For protein determination, the tissues were heated with KOH at 100 ºC and centrifuged at 10,000 X *g* for 10 min. Supernatant was used to estimate the protein level according to the method described by Lowry et al. (1951). For lactate, glucose and ammonia determination, tissue samples were homogenized by adding 10% trichloroacetic acid (1:20 dilution) using a motor-driven Teflon pestle and centrifuged at 1,000 X *g* for 10 min for flocculation of the proteins. The completely deproteinated supernatant was used for lactate determination using the method described by Harrower & Brown (1972), glucose was measured according to Park & Johnson (1949) and ammonia was measured according to Verdouw et al. (1978). For amino acid quantification, tissues (liver and muscle) were twice mechanically disrupted by adding 2 mL phosphate buffer 20 mM, pH 7.5 and the homogenates were centrifuged at 1,000 X *g* for 10 min. The neutral supernatant extracts were used for colorimetric amino acid determination according to Spies (1957). Plasma glucose was measured by the glucose oxidase method with Bioclin test Kit. Plasma was dissolved in 10% trichloroacetic acid (1:20 dilution) and lactate was estimated according to Harrower & Brown (1972). Plasma total protein levels were measured according to Lowry et al. (1951) using bovine serum albumin as standard.

2.8 Statistical procedures

Comparison between two groups was made Student *t*-test. Value of $p \leq 0.05$ was considered statistically significant for all statistical analyses.

3. Results and Discussion

3.1 Acetylcholinesterase enzyme

In both experimental conditions, the acetylcholinesterase (AChE) activity after seven days of exposure to commercial herbicides containing imazethapyr and imazapic increased significantly in brain as compared to the control. In this exposure time, the muscle tissue only showed alterations in laboratory condition, where the AChE activity was decreased (Figure 1). Some effects on the non-target organism are expected, including alterations in AChE activity, especially in medium or long-term of exposure. Brain AChE results obtained in this study are in agreement with those obtained by Miron et al., (2005) where quinclorac (5-20mg/L) and metsulfuronmethyl (400-1200 mg/L) herbicides increased brain AChE activity. These authors also obtained a different result for muscle tissue where AChE activity was reduced. Guimarães et al. (2007) studying *Oreochromis niloticus* exposed to trichlorfon (0.25 ppm) per 96 h, an organo-phosphorate compound (OP), observed a decrease of 85% of the control AChE activity in muscle.

After 30 days of exposure, in field condition the AChE activity in brain was inhibited but in muscle tissue the enzymatic activity was enhanced when compared to fish control. The most commonly found effect of pesticide toxicity is the inhibition of enzyme activity, this inhibition by pesticides was also found in brains of *Anguilla anguilla* by Fernandez-Vega et al. (2002), in *Lepomis macrochirus* by Dutta & Arends (2003), in *Brycon cephalus* by Aguiar et al. (2004), in *Rhamdia quelen* by Miron et al. (2005). However, several pesticides classes have shown enhancement of AChE activity (Miron et al., 2005; Moraes et al., 2007). The enzyme activation is little known in fish.

After long term exposure (90 days), the brain tissue did not show significantly alteration in AChE activity, on the other hand in muscle the AChE activity showed a decrease in activity. Our results are agreement with other authors that found an inhibition in muscle tissue after exposure to different pesticides (Miron et al., 2005; Guimarães et al., 2007). In this study for the authors was evidenced that these

herbicides caused alterations in enzymatic activity of acetilcholinesterase. The combined effects of herbicides seem to be affect cholinergic system and promote undesirable effects on brain and muscle AChE.

3.2 Oxidative stress

In rice field conditions, after seven days of exposure, there was not alteration in oxidative stress parameters such as reactive substances to tiobarbituric acid (TBARS), protein carbonylation and catalase (CAT) and glutathione S-tranferase (GST) enzymes on evaluated tissues. However, in laboratory condition the results showed increase in TBARS levels of brain and white muscle (Table 1). This results are in agreement with those obtained by Ballesteros et al. (2008) that observed enhance in brain TBARS levels after exposure of *Jenynsia multidentata* to endosulfan for a 24-h period. Monteiro et al. (2006) also found an increase of lipid peroxidation in brain tissue of *Brycon cephalus*, after exposure to methyl parathion insecticide. Brain is an organ susceptible to oxidative damages and lipid peroxidation because it has a low antioxidant defense system and high contents of polyunsaturated fatty acids in cell membrane (Matés 2000). There was higher activity of CAT in the liver tissue after exposure of seven days in laboratory (Figure 2). The results found in laboratory could be due to herbicide in this condition being more toxic than rice field exposure. These results agree with results found in other studies (Zhang et al., 2004; Peixoto et al., 2006; Moraes et al., 2007). CAT found mainly in peroxisomes, is associated with elevated concentrations of H_2O_2 because it has important role in removal of hydrogen peroxide which is metabolized to oxygen and water.

After 30 days exposure in rice field, the TBARS levels showed an increase in white muscle, but in cerebral and hepatic tissues alterations were not observed in this parameter. Probably, the increase in muscle TBARS levels is due to the failing of oxidants defenses in this tissue. Differently, occurred increase of protein carbonylation in liver and the GST enzyme activity was decreased (Figures 3 and 4). After 35 days it was not detected herbicides in paddy water, but its effects or its metabolites remained altering and causing disorders in tissues of fish (Figure 5).

After 90 days exposure time, fish continued showing an increase in TBARS levels in brain and white muscle, but in liver continued showing no change. In the same way, in this period, the protein carbonyl levels showed enhance and the GST

activity reduced. The induction of GST is considered beneficial to handle a stress condition, but the reduction of activity is little known. The inhibition of activity in hepatic tissue occurs because liver is one of the first organs exposed to pesticides or other pollutants (Ballesteros et al., 2008). Protein carbonyl formation can occur as a result of oxidative stress and has been shown to play an important role in a various pathological situations (Parvez et al., 2006). This enhancement in protein carbonyl levels in the fish liver exposed for 30 and 90 days indicates that normal protein metabolism was changed, resulting in accumulation of damaged molecules. Our results is in agreement with Parvez & Raisuddin, 2005 that found the protein carbonyl levels increased in liver, kidney, gill and muscle of *Channa punctata* after exposure to deltamethrin, endosulfan or paraquat.

3.3 Metabolic parameters

According to Table 2, after seven days of exposure in field condition the liver tissue showed an increase of protein and ammonia levels indicating favoring protein synthesis and amino acid oxidation. In this same tissue occurred decrease in lactate and glycogen levels after exposure to herbicides in rice field condition. Muscle tissue showed an increase in ammonia, glucose and glycogen, but no alteration in lactate levels. Generally, the reduction of hepatic glycogen indicates stress response caused due to herbicide induced hypoxic condition (Gimeno et al., 1995; Begum, 2004). The elevation in ammonia concentration and decrease of protein levels in muscle of *C. carpio* might be due to increased of protein catabolism. Our results are in disagreement to the results found by Begum (2004) that observed the protein levels enhanced in liver of *C. batrachus* after carbamate exposure. However, the muscle tissue showed a decrease of protein and amino acid levels. High energy demand might have led to the stimulation of protein catabolism (Sancho et al., 1998). Proteins being involved in the architecture and physiology of the cell, they seem to use a key role in cell metabolism. Catabolism of proteins and amino acids play a major role to total energy production in fish (David et al., 2004). The fish increase the storage of glucose throughout muscle glycogen increased. In this period and condition of exposure was evidenced that occurred liver glycogenolysis and protein catabolism.

In laboratory the hepatic tissue showed enhance of glycogen, ammonia and lactate levels and no alteration in protein levels. In the muscle tissue only occurs significantly alterations in glycogen levels, that showed its levels enhanced. Lactate

is the end product of glycolysis under hypoxic conditions, showing that the fish muscle could be experiencing hypoxia because lactate is been produced for the maintenance of the glycogen store. This condition of exposure, the fish needed to obtain quickly energy, because the condition seems more toxic than field condition. The reduction of protein plasmatic in both conditions of exposure indicates the occurred capitation of amino acid through the tissues. Furthermore, the plasma glucose was enhanced in both experiments indicating a response of organism to herbicide toxicity.

After 30 days of exposure in field condition, the liver showed a decrease of ammonia and lactate levels. The ammonia reduction can indicate the maintenance of protein levels while the levels of lactate could be used to maintain the glucose and glycogens levels. The other parameters did not show alterations in this exposure time. In muscle tissue, the protein levels remained reduced and the glycogen enhanced. These results indicate that the protein catabolism continued in this exposure time. Our results are in agreement with those obtained by Glusczak et al. (2007) that also founded the protein levels reduced in muscle of fish exposed to glyphosate per 96 h. The lactate levels showed an increase and glucose levels a decrease indicating anaerobic glycolysis for energy obtaining. The others parameters did not showed alterations. Furthermore, an increase of plasma protein and glucose plasma and a decrease of lactate levels was observed. The high level of blood glucose is a secondary indicator of stress, and the reduction of lactate plasmatic is linked with hepatic drainage of lactate for gluconeogenesis.

After 90 days of exposure, the hepatic tissue showed a decrease in protein and ammonia levels that indicate protein catabolism for defense of organism against herbicide presence. Protein reduction has been observed by others researches showing reduction in fish after exposure to pesticides (Sancho et al., 1998; David et al., 2004). The others parameters did not show alterations. In the muscle tissue, the lactate levels were enhanced, but the amino acid levels were reduced. The changes in lactate level indicate metabolic disorders and the reduction of free amino acids might have been channeled for energy synthesis and other metabolic reactions, but the effects on free amino acids are very variable (Begum & Vijayaraghavan, 1999; Begum, 2004). In this tissue still persists the anaerobic metabolism showing beginning of protein catabolism. The inexistence of alteration in glucose and glycogen stored indicate that the proteins are supplying the muscle metabolism. The

others parameters did not show significantly alterations in this tissue. In the plasma the protein levels were reduced contrasting of what happened with glucose levels that were enhanced. The reduction of protein plasmatic is according with the protein catabolism in tissues and the glucose levels indicate stress caused by herbicide exposure. These results show that the protein and carbohydrate metabolism in the tissues and blood of *C. carpio* were disrupted after exposure to imazethapyr and imazapic herbicides.

4. Conclusion

The present study showed that commercial formulation containing imazethapyr and imazapic herbicides at rice field concentrations may cause changes in toxicology and metabolic parameters of *C. carpio* grown inside the rice paddy. The exposure in laboratory condition can make the herbicide more toxic than exposure in rice field condition. In the field condition, the influence environmental is higher, so the losses of herbicide also are higher. There are long term effects of the herbicide in the fish metabolism. Therefore these parameters can be used to evaluate this herbicide toxicity in fish.

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FIGURE CAPTIONS

Fig.1 Acetylcholinesterase (AChE) activity in brain (A) and muscle (B) tissue of *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.2 Liver tissue catalase (CAT) activity in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.3 Liver protein carbonyl levels in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.4 Liver tissue glutathione S-transferase (GST) activity in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.5 Imazethapyr and imazapic herbicides concentration in water of rice paddy field.

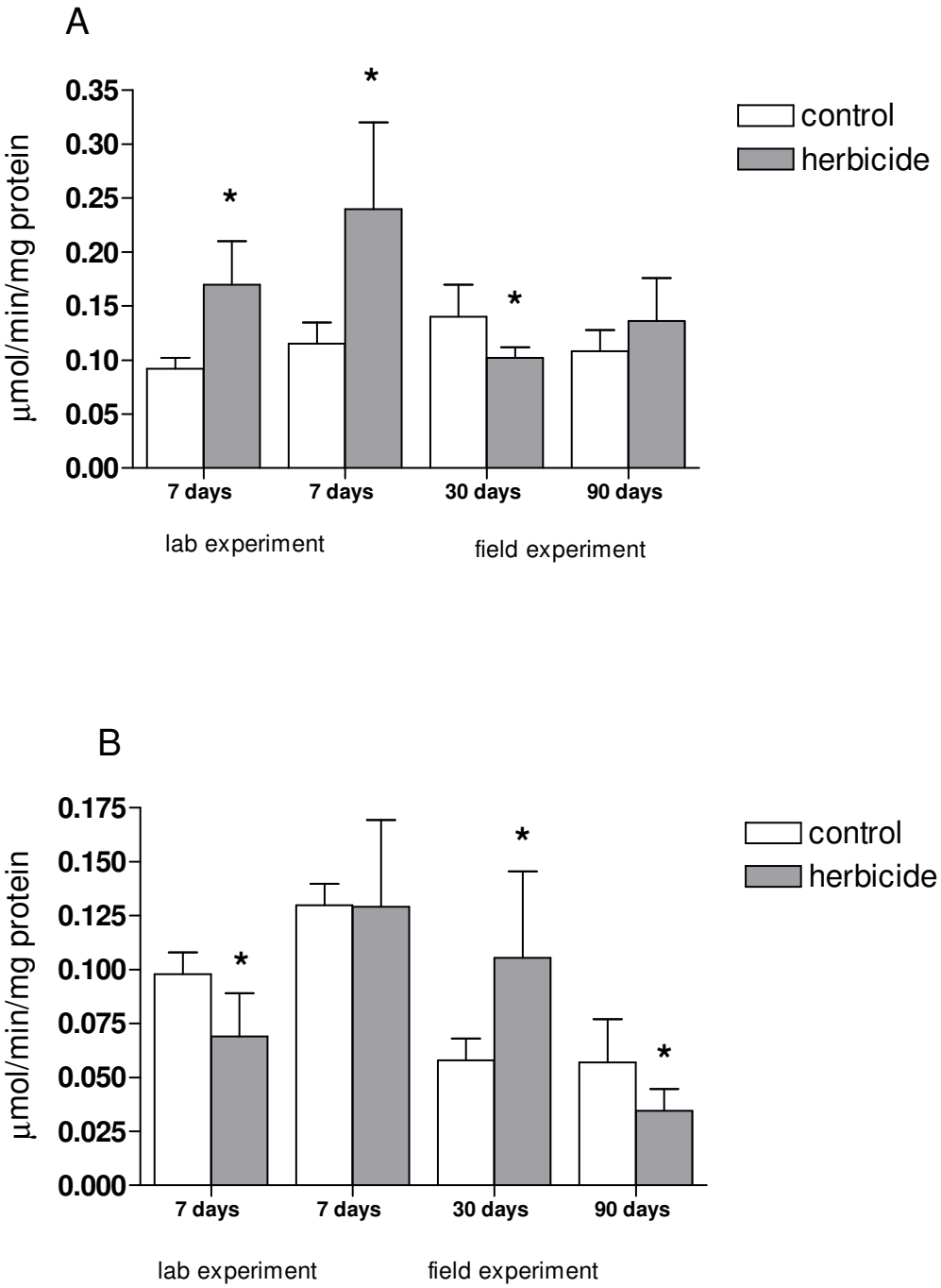

Fig.1 Acetylcholinesterase (AChE) activity in brain (A) and muscle (B) tissue of *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

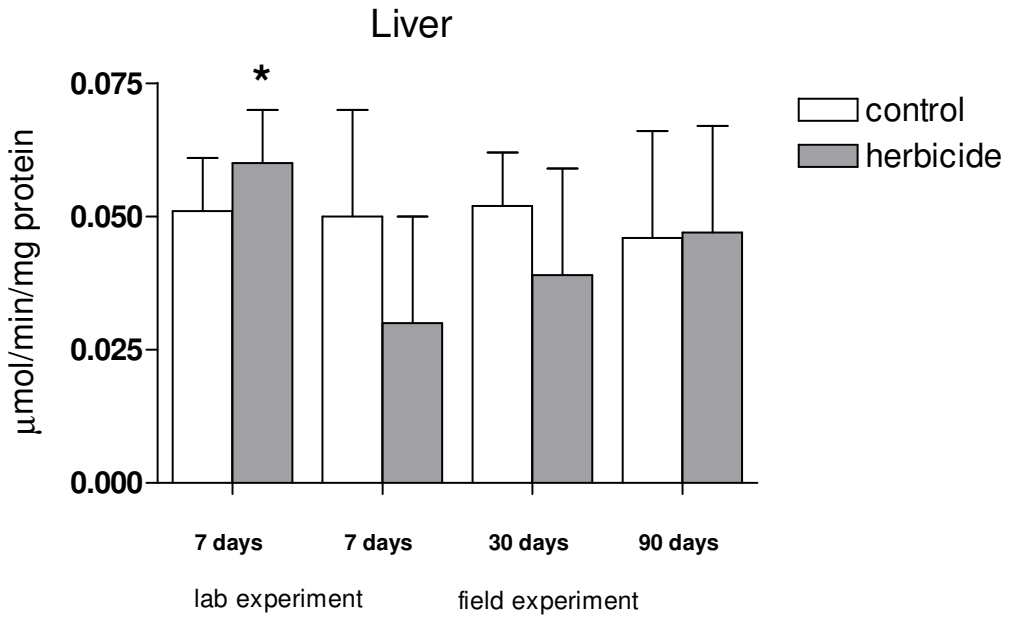

Fig. 2 Liver tissue catalase (CAT) activity in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

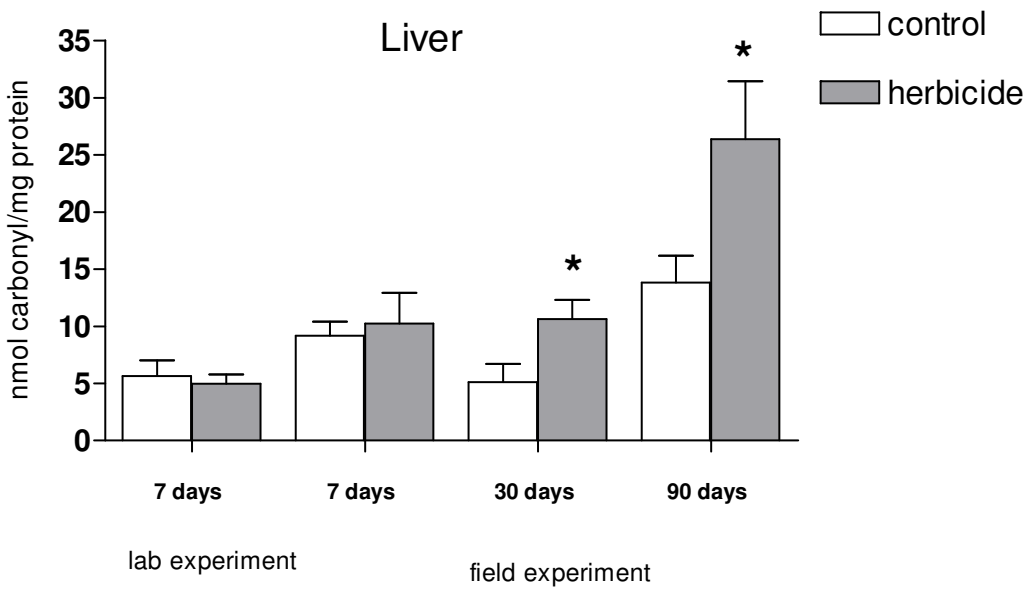

Fig. 3 Liver protein carbonyl levels in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

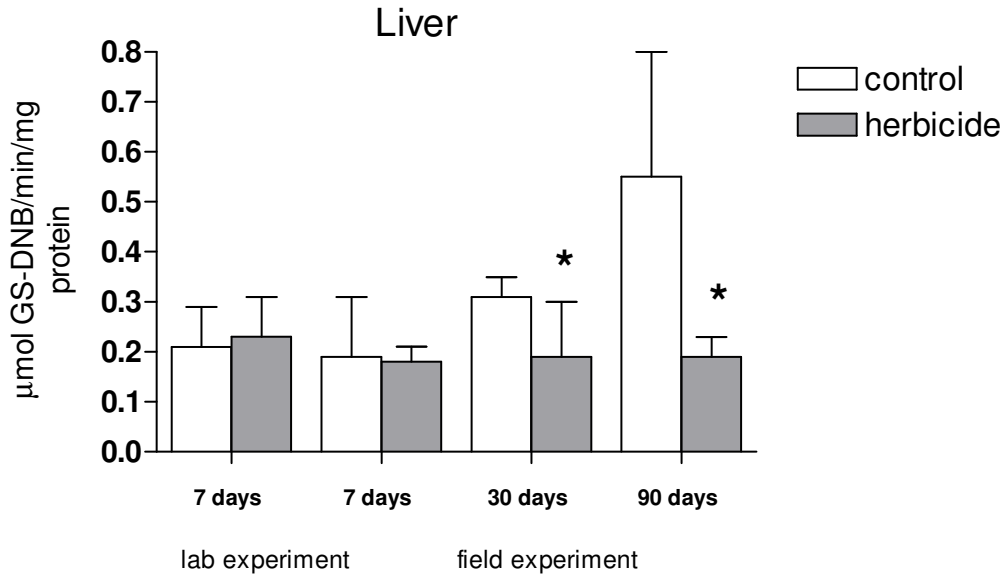

Fig. 4 Liver tissue glutathione S-transferase (GST) activity in *Cyprinus carpio* exposed to commercial herbicide containing imazethapyr and imazapic (0.0075 and 0.0025 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively). *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

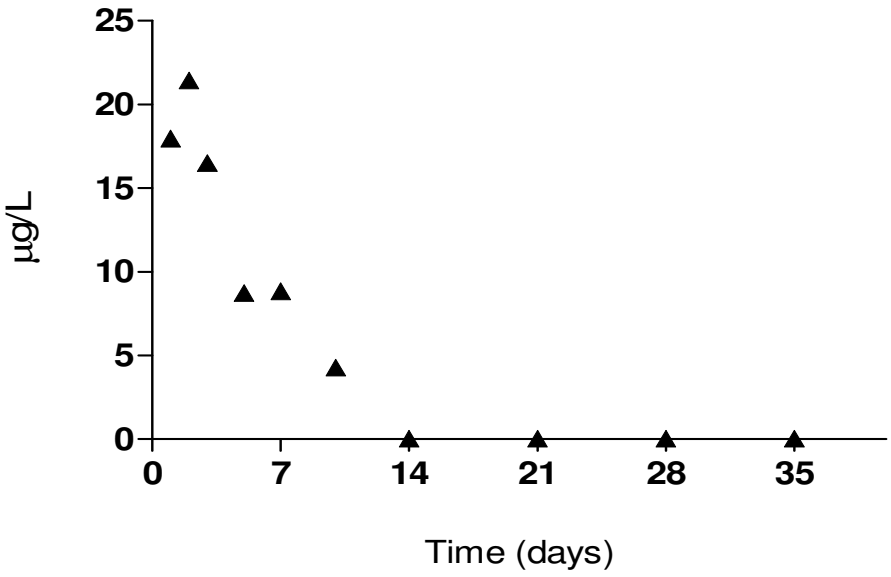

Fig. 5 Imazethapyr and imazapic herbicides concentration in water of rice paddy field.

Table 1: Lipid peroxidation measured throughout TBARS levels (nmol MDA/mg of protein) in brain, liver and muscle of *Cyprinus carpio* exposed to commercial herbicides containing imazethapyr and imazapic at laboratory and rice field condition after 7, 30 or 90 days . Data represent the mean ± SD (n= 20 and n=30 respectively).

Time (days)	Brain		Liver		Muscle	
Laboratory	control	treatment	control	treatment	control	treatment
7	3.315 ± 0.55	4.209 ± 0.86*	1.024 ± 0.25	1.264 ± 0.30	0.495 ± 0.15	0.750 ± 0.13*
Field						
7	3.830 ± 0.49	4.638 ± 0.98	0.926 ± 0.36	1.351 ± 0.46	0.275 ± 0.18	0.121 ± 0.02
30	2.084 ± 0.60	2.754 ± 0.65	1.609 ± 0.71	2.600 ± 0.86	0.256 ± 0.03	0.520 ± 0.2*
90	1.221 ± 0.17	4.930 ± 0.60*	0.664 ± 0.18	0.635 ± 0.15	0.862 ± 0.12	1.833 ± 0.18*

*Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Table 2: Liver, muscle and plasma metabolites of *Cyprinus carpio* exposed to herbicides: imazethapyr and imazapic (0.0075 and 0.0025 mg/L respectivelly) for 7, 30 or 90 days.

Time	Liver		Muscle		Plasma	
Laboratory	control	treatment	control	Treatment	control	Treatment
7 days						
Glycogen	36.1 ± 4.0	56.2 ± 4.9 *	11.2 ± 2.3	12.8 ± 1.9*	NM	NM
Glucose	NM	NM	0.7 ± 0.2	0.6 ± 0.1	38.5 ± 5.5	46.5 ± 8.4*
Lactate	9.3 ± 1.3	13.8 ± 1.1*	29.1 ± 4.4	25.8 ± 2.6	6.5 ± 1.2	6.6 ± 0.8
Protein	123.6 ± 14.7	126.3 ± 6.8	140.4 ± 2.3	143.3 ± 5.4	16.9 ± 1.6	14.9 ± 1.2*
Ammonia	220.1 ± 15.7	283.7 ± 34.1|*	22.5 ± 6.7	23.4 ± 6.2	NM	NM
Amino acids	NM	NM	29.7 ± 6.3	32.1 ± 7.4	NM	NM
Field						
7 days						
Glycogen	91.1 ± 9.4	26.3 ± 6.8*	2.0 ± 0.3	4.0 ± 0.05*	NM	NM
Glucose	NM	NM	0.6 ± 0.09	1.1 ± 0.2*	52.6 ± 11.0	69.0 ± 10.2
Lactate	15.3 ± 2.1	10.5 ± 1.6*	20.2 ± 2.3	21.7 ± 2.2	2.6 ± 0.05	2.0 ± 1.0
Protein	132.8 ± 43.3	263.0 ± 51.1*	188.4 ± 8.2	166.4 ± 5.6*	41.3 ± 3.3	24.3 ± 2.0*
Ammonia	55.8 ± 15.0	91.7 ± 17.4*	28.7 ± 4.3	42.6 ± 3.7*	NM	NM
Amino acids	NM	NM	66.1 ± 6.2	42.8 ± 8.6*	NM	NM
30 days						
Glycogen	45.2 ± 11.9	54.1 ± 16.0	5.2 ± 1.1	7.4 ± 1.9*	NM	NM
Glucose	NM	NM	3.0 ± 0.5	0.7 ± 0.1*	34.2 ± 7.1	61.3 ± 11.6*
Lactate	12.0 ± 0.8	9.3 ± 1.7*	25.5 ± 4.2	34.6 ± 4.0*	3.2 ± 0.4	2.3 ± 0.3*
Protein	182.8 ± 22.5	193.3 ± 56.5	235.4 ± 11.7	221.3 ± 10.2*	41.0 ± 9.0	54.9 ± 5.0*
Ammonia	336.5 ± 54.4	150.8 ± 24.2*	30.2 ± 2.4	27.6 ± 1.7	NM	NM
Amino acids	NM	NM	50.0 ± 4.3	42.3 ± 16.1	NM	NM
90 days						
Glycogen	24.5 ± 5.5	21.6 ± 7.3	6.6 ± 1.5	5.4 ± 2.0	NM	NM
Glucose	NM	NM	0.45 ± 0.1	0.46 ± 0.1	59.3 ± 4.6	107.5 ± 15.2*
Lactate	7.0 ± 1.2	7.6 ± 0.6	27.0 ± 1.9	36.7 ± 4.0*	1.4 ± 0.4	1.8 ± 0.3
Protein	146.4 ± 32.5	103.4 ± 14.8*	470.9 ± 32.4	470.4 ± 24.3	51.2 ± 6.7	41.6 ± 2.8*
Ammonia	220.9 ± 13.4	76.0 ± 5.8*	44.0 ± 9.6	44.2 ± 8.9	NM	NM
Amino acids	NM	NM	59.7 ± 7.2	19.0 ± 3.7*	NM	NM

Glucose, glycogen and lactate in tissue were expressed in μmol/g tissue. Protein was expressed in mg/g tissue or mg/mL plasma, lactate plasma expressed in μmol/mL and glucose in mg/dL plasma. *Indicates significant difference between control and herbicide group ($p \leq 0.05$). NM (not measured).

4.2 Manuscrito 2

Biochemical changes of *Cyprinus carpio* exposed to commercial formulation of clomazone herbicide

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Biochemical changes of *Cyprinus carpio* exposed to commercial herbicide formulation containing clomazone

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Abstract

Pesticides can impact to non-target organism, not only causing death but causing biochemical changes that can affect fish growth and development. For this reason a field and a laboratory experiment were carried out to evaluate toxicological responses of *Cyprinus carpio* exposed to a commercial herbicide formulation containing clomazone (500 mg/L). Fish were exposed either at laboratory and field conditions to a correspondent to 0.5 mg/L of the formulated herbicide. At laboratory conditions, fish were exposed for seven days and at field conditions, they were exposed for 7, 30 or 90 days. After exposure, toxicological and metabolic parameters were analyzed in different fish tissues. Fish exposed seven days at rice field condition did not showed alteration in AChE activity, but in laboratory condition, in the same period of exposure, it was observed a decrease in muscle AChE activity. After 30 and 90 days in field condition, the AChE activity did not change. At same evaluation period, the oxidative stress parameters were changed in both conditions. The metabolic parameters showed changes in both conditions and at all exposure period. After 30 days of exposure in field condition, the AChE enzyme did not show alterations. Both stress oxidative and metabolic parameters were altered in this condition of exposure. After 90 days of exposure in field, the AChE activity also did not show alterations. The disorders in oxidative stress parameters and metabolism remained in different tissues during 90 days. These overall results indicate that AChE activity change only in laboratory condition and oxidative stress together with metabolic parameters may be good indicators of herbicide contamination in *C. carpio* at rice field condition.

KeyWords: *Cyprinus carpio*, oxidative stress, AChE, metabolism, herbicide.

1. Introduction

Several environmental pollutants can cause alterations in biochemical parameters of non-target organisms. Pesticide is one of the chemicals known of affecting fish (Moraes et al., 2007). Furthermore, the contaminants enhance the intracellular formation of reactive oxygen species (ROS). ROS are formed and degraded by all aerobic organisms (Nordberg & Arnér, 2001). When an imbalance occurs between ROS and antioxidant system, the cell can development oxidative stress.

Oxidative stress in fish also is represented by damage in biological systems or a failure in antioxidant defense system. The hydroxyl radical ($OH^{\bullet}$), that also is a product of reactions with free radicals, react quickly with near molecules leading to oxidative changes in proteins, lipids and nucleic acid (Cardoso et al., 2006). Lipid peroxidation (LPO) and carbonylation of proteins has been used to assess the effect of pollutants in aquatic organisms. Different studies showed that when these parameters are enhanced, the fish are under oxidative stress (Parvez et al., 2006; Almroth et al., 2008). In fish, the antioxidant system includes enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx) and others. An increase in their activities can indicate disorders that could be undesirable. The CAT and GST enzymes have been used as a parameter to assess environmental pollutant affecting fish, including the herbicides (Moraes et al., 2007; Zhang et al., 2008).

Another enzyme that has been used to assess exposure of several contaminants is acetylcholinesterase (AchE). Herbicides can cause changes in enzyme activity, either inhibition or activation, but the activation effects are little known (Miron et al., 2005; Glusczak et al., 2006). Clomazone herbicide enhanced muscle AChE activity of *Leporinus obtusidens* after long term exposure (30 days) (Moraes et al., 2007). AChE is a key enzyme in cholinergic transmission in the nervous system. The widely enzyme function is catalyze the hydrolysis of ACh into acetate and choline in the synaptic cleft (Yi et al., 2006).

Pesticides can also cause disorders in carbohydrate, protein metabolism and blood parameters (Crestani et al., 2006; Glusczak et al., 2007). These parameters

are secondary indicators of stress, thus when are assessed together with the others parameters, can give a good picture of fish health condition.

Cyprinus carpio (Cyprinidae) was chosen for this research, since the effect of clomazone on fish species, particularly on this one, is scarcely studied. It was also chosen because it is a commercially relevant species for fisheries in the southern region of Brazil. This specie is very resistant and adaptable to different temperatures (Castagnolli & Cyrino, 1986).

Clomazone {2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone}, is a widely used herbicide for weed control in rice on Rio Grande do Sul State, Southern Brazil. It has high water solubility (1100 mg/L), and its half-live in soil is 24 days (Senseman, 2007). This herbicide has been reported contaminating surface water in this state (Marchezan et al. 2007), and has been reported affecting different fish species (Miron et al., 2005; Crestani et al., 2006; Moraes et al., 2007).

The effect of commercial formulation used in rice field condition is scarcely studied in this species of fish. Thus, the present study aimed to investigate the effects of field concentration of a commercial formulation of rice herbicide containing clomazone in *Cyprinus carpio*, and to determine the possible indicators of fish exposure to this herbicide.

2. Materials and Methods

2.1 Fish

Cyprinus carpio of both genders weighting of 20.0 ± 1.0 g and measuring 11.0 ± 1.0 cm length, were obtained from a fish farm (RS, Brazil). Fish were acclimated to laboratory conditions for 10 days, in tanks (250 L) prior to the experiments. They were kept in continuously aerated water with a static system and with a natural photoperiod (12h light/12h dark). After this period, fish were divided in two groups, one group was transferred to rice paddy and the other was transferred to laboratory tanks, with different exposure time, at rice paddy condition they were exposed for 7, 30 and 90 days and at laboratory for seven days. The rice paddy water, during experimental period (90 days) had the following average parameters: temperature 24.5 ± 2.0 ^{o}C, pH 6.5 ± 0.2 units, dissolved oxygen 4.21 ± 2.0 mg/L, nonionized ammonia 0.8 ± 0.01 μg/L, nitrite 0.06 ± 0.01 mg/L. In laboratory conditions the

average of water parameters were: temperature 22.1 ± 1.0 °C, pH 7.7 ± 0.2 units, dissolved oxygen 7.3 ± 1.0 mg/L, nonionized ammonia 0.3 ± 0.01 μg/L, nitrite 0.04 ± 0.01 mg/L. Fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil) both during acclimation and exposure period.

2.2 Chemicals

A commercial formulation of the herbicide clomazone (Gamit - FMC) was used in both experiments. Acethylthiocholine (ASCh), 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) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3 Experimental design

2.3.1 Field experimental

Fish were allocated in 2 groups (in triplicate) of 10 animals distributed per tank (30 fish per treatment). One group was the control fish and the other group was the exposed fish to the herbicide, with initial concentration corresponding to 0.5 mg/L of clomazone, for 7, 30 or 90 days period. The experiment was carried out in the rice paddy field, with the fishes trapped in submersed cages, measuring 0.30 m (diameter) x 1.05 m (length). Herbicide concentration in water was monitored from the first day until it was not detected. Herbicide was analyzed by High Pressure Liquid Chromatography (HPLC) using the method described by Zanella et al. (2002). After each exposure timing (7, 30 or 90 days), a sample of 10 individuals were taken from the tanks and then submitted to blood and tissues (brain, liver and white muscle) collection.

2.3.2 Laboratory experiment

This experiment was carried out in tanks of 30 L capacity (in duplicate) and each tank had 10 fish. The herbicide concentrations were the same used in the field condition. The herbicide was added only in beginning of experiment without water or herbicide replacement. To do so, it was used a filter (with wool acrylic) in each tank to remove feeding waste. These filters were cleaned every day. After the experimental

period, the fish were sampled and blood and tissues were collected (brain, liver and white muscle).

The AChE (EC 3.1.1.7) activity was measured using the method described by Ellman et al. (1961) and modified by Miron et al. (2005). Brain and muscle tissues (30 mg) were weighted and homogenized in a Potter-Elvejhem glass/Teflon homogenizer with sodium phosphate buffer 50 mM pH=7.2 and Triton X-100 1%. The homogenate was than centrifuged for 10 minutes at 3,000 X *g* at 5°C and the supernatant was used as enzyme source. Aliquots of supernatant (50 and 100 µL) (brain and muscle, respectively) were incubated at 30°C for two minutes with a solution containing 0.1 M sodium phosphate buffer pH 7.5 and 1 mM DTNB. After the incubation period, the reaction was initiated by the addition of ASCh (0.5 mM). The final volume was 2.0 mL. Absorbance was measured by spectrophotometry (Femto Scan spectrophotometer) at 412 nm during 2 min. Enzyme activity was expressed as µmol of ASCh hydrolyzed/min/mg protein.

2.4.2 Catalase activity assay

Catalase (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry (Nelson & Kiesov 1972). Liver tissue were homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.5 (1:20 w/v), centrifuged at 10,000 X *g* for 10 min at 4°C. The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.05 mL homogenate. Change of H_2O_2 absorbance in 60 s was measured by at 240 nm. Catalase activity was calculated and expressed in µmol/min/mg protein.

2.4.3 Glutathione S-tranferase (GST)

GST activity was measured according to Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The formation of S-2, 4-dinitrophenyl glutathione was monitored by the increase in absorbance at 340 nm against blank. The extinction coefficient used for CDNB was 9.6 mM/cm. The activity was expressed as µmol GS-DNB/min/mg protein.

2.5 Carbonyl assay

The liver tissue was homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed

by the method described by Yan et al. (1995) with some modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid. After incubation at room temperature for one hour in dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane (99.5%) and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40s and centrifuged at 10,000 X *g* for 15 min. Then, the protein isolated from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1), and suspended in 1 mL of denaturing buffer and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was performed in duplicate and two tubes blank incubated with 2 N HCl without DNPH was included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22,000 M/cm.

2.6 Lipid peroxidation estimation assay

Lipid peroxidation was estimated by a TBARS assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured. Liver, muscle and brain homogenates (100-400 µL) were added TCA 10% and 0.67% thiobarbituric acid were added to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a micro-centrifuge tube and incubated for 15 min at 95 ºC. After cooling, it was centrifuged at 5,000 X *g* for 15 min and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmols MDA/mg protein according to Buege & Aust (1978).

2.7 Protein determination

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

2.8 Metabolic parameters

Liver and muscle glycogen were determined by the method described by Bidinotto et al. (1998) after KOH (6N) and ethanol addition for hydrolysis and precipitation of glycogen. For protein determination, the tissues were heated with KOH at 100 ºC and centrifuged at 10,000 X *g* for 10 min. Supernatant was used to estimate the protein level according to the method described by Lowry et al. (1951).

For lactate, glucose and ammonia determination, tissue samples were homogenized by adding 10% trichloroacetic acid (1:20 dilution) using a motor-driven Teflon pestle and centrifuged at 1,000 X *g* for 10 min for flocculation of the proteins. The completely deproteinated supernatant was used for lactate determination using the method described by Harrower & Brown (1972), glucose was measured according to Park & Johnson (1949) and ammonia was measured according to Verdouw et al. (1978). For amino acid quantification, tissues (liver and muscle) were twice mechanically disrupted by adding 2 mL phosphate buffer 20 mM, pH 7.5 and the homogenates were centrifuged at 1,000 X *g* for 10 min. The neutral supernatant extracts were used for colorimetric amino acid determination according to Spies (1957). Plasma glucose was measured by the glucose oxidase method with Bioclin test Kit. Plasma was dissolved in 10% trichloroacetic acid (1:20 dilution) and lactate was estimated according to Harrower & Brown (1972). Plasma total protein levels were measured according to Lowry et al. (1951) using bovine serum albumin as standard.

2.8 Statistical procedures

Comparison between two groups was made Student *t*-test. Value of $p \leq 0.05$ was considered statistically significant for all statistical analyses.

3. Results and Discussion

3.1 Oxidative stress

At seven days of exposure in field condition, the TBARS levels were enhanced in liver and brain (Table 1). This parameter did not change in muscle tissue. Lipid peroxidation (LPO) has often been used as an effective biomarker of toxic pollutants in fish (Livingstone, 2001). LPO has usually been indicated by TBARS in fish (Crestani et al., 2007; Glusczak et al., 2007). The others parameters such as protein carbonilation, CAT and GST did not show alterations in field condition (Figures 1, 2 and 3).

In laboratory, in the same exposure period, the liver, brain and muscle showed increase in TBARS levels. The status of oxidative stress remains in this tissues. In liver, the carbonyl levels were decreased and the CAT activity was increased

(Figures 1 and 2). The decrease in protein carbonyl levels according some authors may indicate that the susceptibility to protein degradation has been increased by mild oxidation of proteins (Almroth et al., 2005). Parvez et al. (2006) also found reduction in protein carbonyl levels in liver, kidney and gills of *Wallago attu* exposed to several pollutants in a site of river Yamuna. CAT is a very important enzyme of antioxidant system that removes the hydrogen peroxide which is metabolized to oxygen and water (Peixoto et al., 2006). Our study showed that CAT activity in liver was increased probably in response to higher hepatic TBARS levels; this is a typical response against herbicide toxicity. A significantly increased CAT activity was also observed in some studies after the exposure to different pollutants and pesticides (Ahmad et al., 2000; Zhang et al., 2004; Peixoto et al., 2006; Moraes et al., 2007). When *Leporinus obstusidens* were exposed at rice paddy field to clomazone and propanil the catalase activity enhanced (Moraes et al., 2007). GST enzyme did not show alteration in this condition of exposure (Figure 4).

After 30 days in field condition, the TBARS levels showed an increase in white muscle, but in cerebral tissue a decreased was observed. Lipid peroxidation is one of the main processes induced by oxidative stress (Oruç & Usta, 2007). Considering that LPO is considered a valuable indicator of oxidative damage of cellular components, our results suggest that the increase of TBARS in white muscle of *Cyprinus carpio* after exposure to clomazone indicates oxidative stress in this tissue and that antioxidant defenses were not totally able to effectively scavenge them, thus leading to LPO. In this same period, the protein carbonyl levels enhanced. The protein carbonylation resulting in protein oxidation could be linked to TBARS increase due to ROS could directly attack protein and lead to the carbonyl formation. Some authors also consider this hypothesis (Bainy et al., 1996; Zhang et al., 2008). Our results are in agreement with Parvez & Raisuddin, (2005) that observed an enhanced in protein carbonyl levels of fish (*Channa punctata*) exposed to different pesticides. An increase in protein carbonyl levels would indicate that normal protein metabolism was disrupted (Almroth et al., 2005). GST is the important enzyme involved in catalyzing the conjugation of a wide variety of electrophilic substrates to reduced glutathione and protects the cell against effects of xenobiotics (Ferrari et al., 2007). In our work, GST activity showed a significantly reduction in liver after exposure to commercial formulation of clomazone (Figure 3).

After 90 days, the TBARS levels increase in brain and remained enhanced in muscle tissue, indicating that a condition of stress remained in this fish species after long term exposure. The carbonyl protein was increased in liver, due to an oxidative stress status. This parameter serves as a good biomarker for oxidative stress, especially in environmental contamination context. The activity of catalase enzyme was significantly decreased in the liver of fish exposed to commercial formulation containing clomazone. Our results are in agreement with Dorval & Hontela (2003) that found CAT activity reduced in *Oncorhynchus mykiss* after exposure to endosulfan. Ballesteros et al. (2008) also observed a decrease in CAT activity in liver of *Jenynsia multidentata* after to endosulfan insecticide exposure. The GST enzyme remained inhibited in the hepatic tissue of *C. carpio* exposed to clomazone herbicide after 90 days.

3.2 Acetylcholinesterase enzyme

After seven days of exposure in field condition, the AChE activity was not changed in brain and muscle (Figure 4). But in laboratory condition, AChE enzyme in the muscle tissue showed an inhibition. In this condition, the brain also did not showed alterations. In laboratory condition, Crestani et al. (2007) found that clomazone herbicide is a potent brain and muscle AChE inhibitor of *Rhamdia quelen*, reaching the maximum of 45-47% of inhibition at concentrations of 0.5 or 1.0 mg/L. Another study indicates that higher concentrations (5, 10 or 20 mg/L) of a commercial formulation clomazone also in laboratory conditions caused inhibition in AChE activity in brain and muscle of silver catfish (Miron et al., 2005). Similar results were found when *Oreochromis niloticus* exposed to an organophosphate insecticide (trichlorfon), showed AChE activity decreased in muscle tissue (Guimarães et al., 2007). The results of present work showed that AChE activity in carps could be more resistant to disruption by clomazone and the rice field rate is not enough to cause alterations in this enzyme.

In field condition, the AChE did not changed in both tissues after 30 and 90 days of exposure. In another rice paddy experiment, a 30-days exposure of *Leporinus obtusidens* to clomazone (0.5 mg/L), showed inhibition of activity in brain and also increase of activity in muscle (Moraes et al., 2007). In other study with 90 days of exposure the *L. obtusidens* showed a reduction in AChE activity in both tissues (brain and muscle) after clomazone exposure (unpublished data).

The inhibition or activation of AChE can affect the process of cholinergic neurotransmission and promote undesirable effects in fish. The reduction of muscle AChE activity found in our investigation may be caused by the herbicide molecule and/or the adjuvants used in the formulation. The absence of effects could indicate that brain and muscle of carps are poorly affected by commercial formulation containing clomazone.

3.3 Metabolic parameters

After seven days of exposure, in both conditions, the liver and muscle tissues showed an increase of glycogen (Table 2). These results are in disagreement with others studies that have shown glycogen consume in different tissues as result of the stress response caused by pesticides exposure (Begum & Vijayarghavan, 1995; Sancho et al., 1998; Aguiar et al., 2004; Glusczak et al., 2006; Fonseca et al., 2008). However, Crestani et al (2006) also found the hepatic glycogen levels increased in *Rhamdia quelen* exposed to clomazone herbicide. The glycogen levels in tissues of *C. carpio* can suggest that this fish stores glycogen as energy source to compensate stress situation. Muscular glucose levels were increased after exposure in field condition and reduced after exposure in laboratory condition.

In field condition, the lactate levels did not changed in liver and was enhanced in muscle indicating an anaerobic metabolism by increase of lactate to maintain glucose reserves as liver glycogen and muscle glucose. In laboratory condition, liver showed an increase of lactate levels and in muscle no alteration was observed. According to Glusczak et al. (2006), the elevation of lactate also indicates metabolic disorders and a clear response against energy depletion.

Protein levels increased in liver of fish exposed in field condition and in muscle did not change. But in laboratory condition protein levels did not change in both tissues. This enhance of protein levels in liver can be indicating a favoring protein synthesis and amino acid oxidation. Crestani et al. (2006) also found protein levels increased in liver of *Rhamdia quelen* after 192 h of exposure to clomazone herbicide. After exposure in field condition, the amount of ammonia in liver was increased and in muscle did not change. After laboratory exposure, the amount of ammonia was unaltered in liver and enhanced in muscle tissue. Our results are in agreement with those obtained by Glusczak et al. (2006, 2007) where the fish

exposed to glyphosate herbicide showed an increase in ammonia levels in liver and muscle. In both experimental conditions, the amino acids levels were reduced in muscle tissue. In plasma occurred a decreased in protein in both conditions of exposure. The lactate levels were increased in plasma after exposure in laboratory condition and decreased in plasma of fish exposed in field condition. The reduction of lactate in plasma is related with hepatic drainage of lactate to maintain hepatic gluconeogenesis. In both conditions of exposure occurred an enhanced in plasmatic glucose levels. The high level of blood glucose is a secondary indicator of stress (Table 2).

After 30 days of exposure, the amount of hepatic and muscular glycogen did not change. In liver was observed a reduction in lactate levels and in muscle this parameter was modified. This decrease may indicate higher gluconeogenesis adaptation. Fonseca et al. (2008) also observed a decreased of lactate in liver and muscle of *Leporinus obtusidens* after exposure to 2,4-D herbicide. In liver, the protein levels were decreased and in muscle no occurred alteration. In both tissues, occurred a reduction in ammonia levels. The glucose levels were decreased in muscle of *C. carpio* exposed to clomazone. In plasma the lactate and glucose levels were enhanced showing that the disorder remains in metabolism of fish exposed to herbicide.

After 90 days of exposure, in liver occurred an increase in glycogen levels and a reduction in ammonia levels, but no alteration in others parameters. The glycogen results indicate that the fish remain using this metabolite as energy source to compensate stress situation. In muscle tissue, only there was a decrease in amino acid levels. In plasma remained a reduction in protein levels and a hyperglycemia situation. These results show that the protein oxidation persists and hyperglycemia situation indicated a long time response to clomazone toxicity.

4. Conclusion

The present study showed that commercial herbicide formulation containing clomazone at paddy rice field concentrations may cause changes in toxicological and metabolical parameters of *C. carpio* grown inside the rice paddy. The results obtained in laboratory condition were very similar to those observed at field condition. There are long term effects of the herbicide in the fish metabolism. Therefore these parameters can be used to evaluate clomazone toxicity in fish.

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FIGURE CAPTIONS

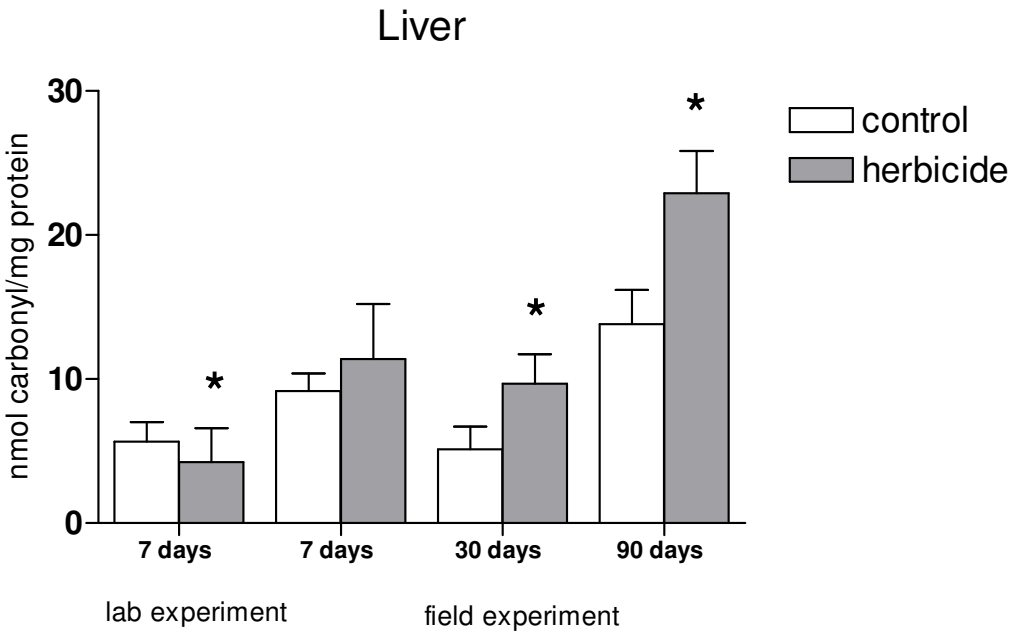
Fig.1 Liver protein carbonyl levels in *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.2 Liver tissue catalase (CAT) activity in *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.3 Liver tissue glutathione S-transferase (GST) activity in *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.4 Acetylcholinesterase (AChE) activity in brain (A) and muscle (B) tissue of *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Fig.5 Clomazone concentration in water of rice paddy field.



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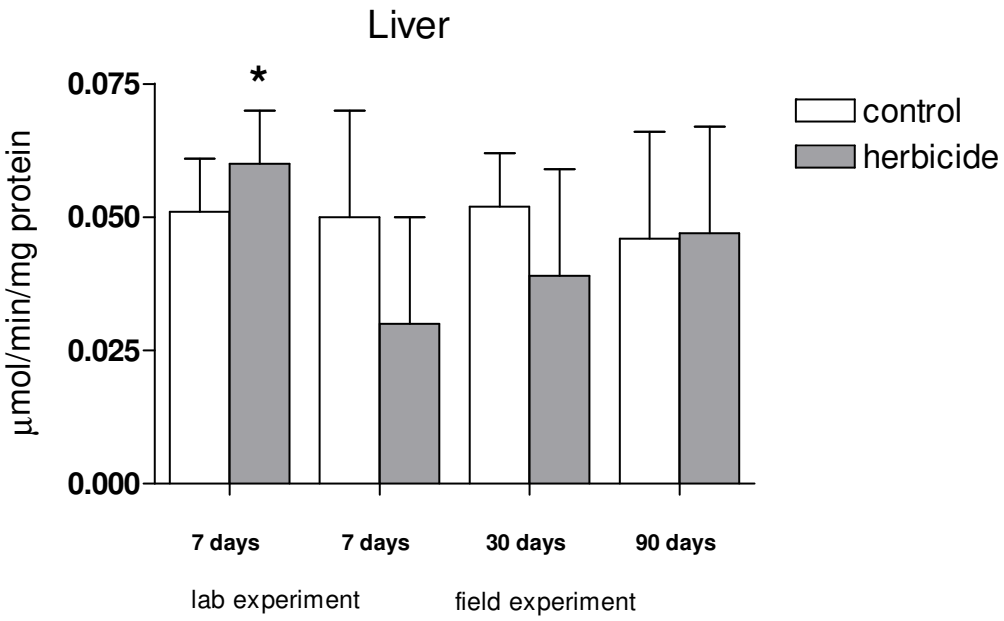

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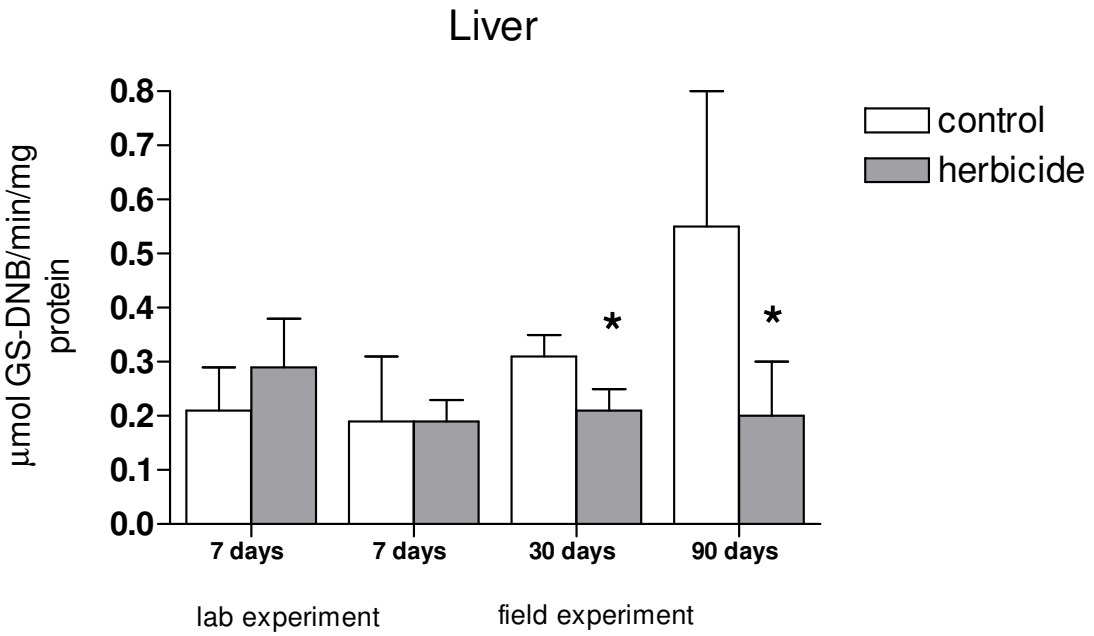

Fig.3. Liver tissue glutathione S-transferase (GST) activity in *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

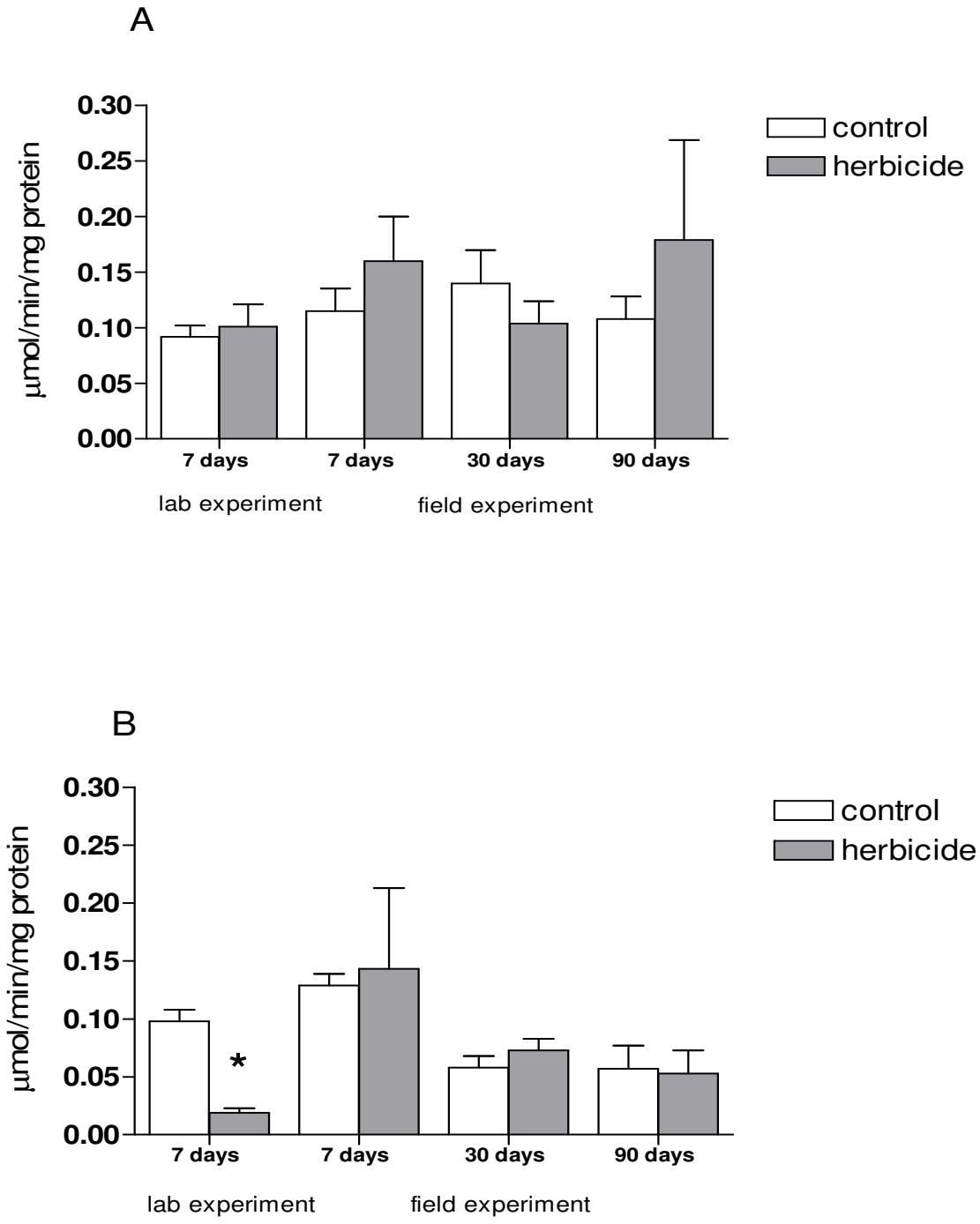

Fig.4. Acetylcholinesterase (AChE) activity in brain (A) and muscle (B) tissue of *Cyprinus carpio* exposed to commercial herbicide containing clomazone (0.5 mg/L) at laboratory (7 days) and rice field condition after 7, 30 or 90 days. Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively) *Indicates significant difference between control and herbicide group ($p \leq 0.05$).

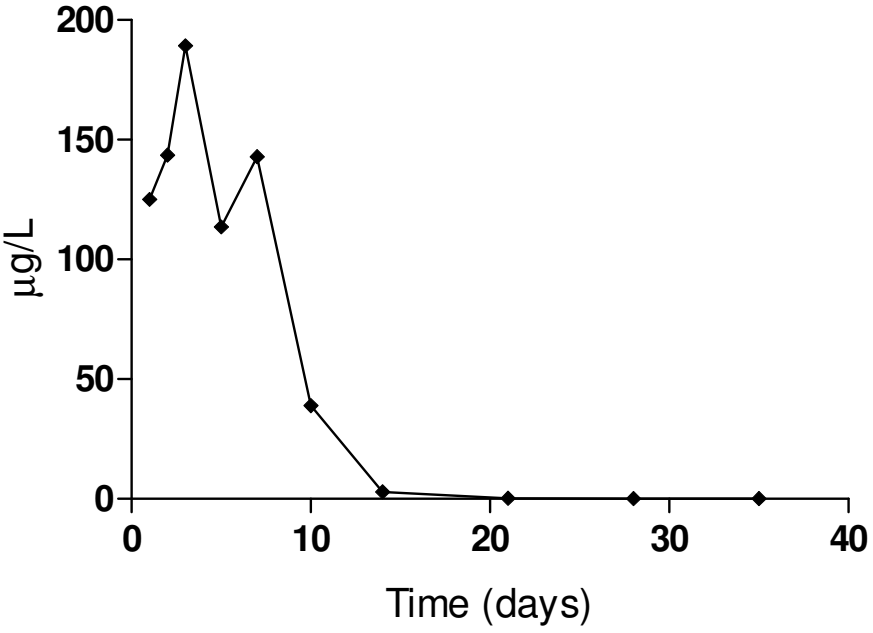

Fig.5. Clomazone concentration in water of rice paddy field.

Table 1: Lipid peroxidation measured throughout TBARS levels (nmol MDA/mg of protein) in brain, liver and muscle of *Cyprinus carpio* exposed to commercial herbicide containing clomazone at laboratory (7 days) and rice field condition after 7, 30 or 90 days . Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively).

Time (days)	Brain		Liver		Muscle	
Laboratory	control	treatment	control	treatment	control	Treatment
7	3.315 ± 0.55	4.425 ± 0.73*	1.024 ± 0.25	1.263 ± 0.18*	0.495 ± 0.15	0.750 ± 0.21*
Field						
7	3.830 ± 0.49	5.906 ± 0.86*	0.926 ± 0.36	3.756 ± 1.31*	0.275 ± 0.18	0.267 ± 0.13
30	2.084 ± 0.60	1.337 ± 0.20*	1.609 ± 0.71	1.828 ± 0.53	0.256 ± 0.03	0.429 ± 0.13*
90	1.221 ± 0.17	2.535 ± 1.12*	0.664 ± 0.18	0.596 ± 0.17	0.862 ± 0.12	1.410 ± 0.29*

*Indicates significant difference between control and herbicide group ($p \leq 0.05$).

Table 2: Liver, muscle and plasma metabolites of *Cyprinus carpio* exposed to commercial herbicide containing clomazone at laboratory (7 days) and rice field condition after 7, 30 or 90 days . Data represent the mean ± SD (n= 20 and n=30, laboratory exposure and field exposure, respectively).

Time	Liver		Muscle		Plasma	
Laboratory	control	treatment	control	treatment	Control	Treatment
7 days						
Glycogen	36.1 ± 4.0	138.1 ± 20.7 *	11.2 ± 2.3	13.6 ± 2.4*	NM	NM
Glucose	NM	NM	0.7 ± 0.2	0.6 ± 0.1*	38.5 ± 5.5	60.5 ± 7.9*
Lactate	9.3 ± 1.3	12.9 ± 1.0*	29.1 ± 4.4	26.9 ± 3.6	6.5 ± 1.2	10.6 ± 1.5*
Protein	123.6 ± 14.7	117.9 ± 5.2	140.4 ± 2.3	138.7 ± 8.7	16.9 ± 1.6	11.8 ± 1.8*
Ammonia	220.1 ± 15.7	235.2 ± 34.1|	22.5 ± 6.7	31.6 ± 12.2*	NM	NM
Amino acids	NM	NM	29.7 ± 6.3	20.3 ± 7.7*	NM	NM
Field						
7 days						
Glycogen	91.1 ± 9.4	140.6 ± 10.9*	2.0 ± 0.3	3.4 ± 0.27*	NM	NM
Glucose	NM	NM	0.6 ± 0.09	2.1 ± 0.2*	52.6 ± 11.0	68.0 ± 8.6*
Lactate	15.3 ± 2.1	16.4 ± 1.3	20.2 ± 2.3	23.0 ± 1.5*	2.6 ± 0.05	1.8 ± 0.7*
Protein	132.8 ± 43.3	314.5 ± 36.5*	188.4 ± 8.2	183.7 ± 5.6	41.3 ± 3.3	28.4 ± 2.8*
Ammonia	55.8 ± 15.0	178.0 ± 24.4*	28.7 ± 4.3	32.2 ± 3.0	NM	NM
Amino acids	NM	NM	66.1 ± 6.2	42.3 ± 9.1*	NM	NM
30 days						
Glycogen	45.2 ± 11.9	52.7 ± 14.4	5.2 ± 1.1	6.6 ± 1.3	NM	NM
Glucose	NM	NM	3.0 ± 0.5	0.7 ± 0.1*	34.2 ± 7.1	55.5 ± 15.9*
Lactate	12.0 ± 0.8	9.4 ± 1.2*	25.5 ± 4.2	24.6 ± 1.5	3.2 ± 0.4	4.3 ± 0.4*
Protein	182.8 ± 22.5	130.6 ± 12.4*	235.4 ± 11.7	238.0 ± 27.7	41.0 ± 9.0	47.9 ± 10.0
Ammonia	336.5 ± 54.4	153.8 ± 16.6*	30.2 ± 2.4	26.2 ± 2.3*	NM	NM
Amino acids	NM	NM	50.0 ± 4.3	47.1 ± 3.6	NM	NM
90 days						
Glycogen	24.5 ± 5.5	37.9 ± 12.8*	6.6 ± 1.5	6.9 ± 1.8	NM	NM
Glucose	NM	NM	0.45 ± 0.1	0.51 ± 0.2	59.3 ± 4.6	100.5 ± 14.2*
Lactate	7.0 ± 1.2	8.1 ± 0.9	27.0 ± 1.9	27.7 ± 4.5	1.4 ± 0.4	1.7 ± 0.5
Protein	146.4 ± 32.5	117.6 ± 21.6	470.9 ± 32.4	470.9 ± 35.3	51.2 ± 6.7	38.5 ± 6.3*
Ammonia	220.9 ± 13.4	169.4± 8.8*	44.0 ± 9.6	44.1± 4.9	NM	NM
Amino acids	NM	NM	59.7 ± 7.2	48.7 ± 9.0*	NM	NM

Glucose, glycogen and lactate in tissue were expressed in µmol/g tissue. Protein was expressed in mg/g tissue or mg/mL plasma, lactate plasma expressed in µmol/mL and glucose in mg/dL plasma. *Indicates significant difference between control and herbicide group ($p \leq 0.05$). NM (not measured).

5 DISCUSSÃO

Neste estudo, após sete dias de exposição, em ambas as condições experimentais, observou-se um aumento da atividade da enzima acetilcolinesterase (AChE) em cérebro de carpas expostas a formulação comercial que contém os herbicidas imazetapir e imazapic. O herbicida clomazone não alterou a atividade da enzima neste tecido. No músculo, a atividade da AChE foi reduzida após a exposição aos dois herbicidas comerciais em condições de laboratório. No campo, após 30 dias de exposição, ocorreu uma diminuição da atividade da enzima em cérebro e um aumento no músculo de peixes expostos a formulação contendo imazetapir e imazapic. Aos 90 dias de exposição, o tecido muscular mostrou novamente uma redução da atividade da AChE. O herbicida clomazone não causou alteração em ambos tecidos após 30 e 90 dias. O efeito de exposição a pesticidas mais comumente encontrado é a redução da atividade da AChE (GLUSCZAK *et al*., 2007; FONSECA *et al*., 2008). Miron *et al*. (2005) encontraram um aumento na atividade da AChE em cérebro de jundiás expostos por 96 h aos herbicidas quinclorac e metasulfuron-metil (100-400 e 400-1200 mg/L, respectivamente). Porém, neste mesmo trabalho foi observada a redução da atividade da enzima em cérebro de peixes expostos ao herbicida clomazone (5-20 mg/L) e no músculo destes peixes após a exposição aos herbicidas quinclorac, metasulfuron-metil e clomazone. Moraes *et al*., (2007) observaram um aumento da atividade da AChE em músculo de piavas expostas por 30 dias aos herbicidas clomazone (0,5 mg/L), propanil (3,6 mg/L) e metasulfuron-metil (0,002 mg/L). Além disso, neste mesmo estudo uma redução da atividade foi observada em cérebro de piavas expostas aos herbicidas clomazone e quinclorac (0,5 e 0,375 mg/L respectivamente). No presente estudo, aos 30 dias de exposição em campo, não foram encontrados resíduos de ambas as formulações comerciais estudadas, mas as desordens sobre a atividade da enzima permaneceram mesmo após longo tempo de exposição (90 dias). O efeito da formulação comercial que contém o herbicida clomazone persiste sobre a atividade da enzima acetilcolinesterase após o período (21 dias) em que os herbicidas foram detectados na água. Os efeitos sobre a enzima foram variados, dependendo do período estudado, tecido considerado e também do herbicida

testado. Na literatura existem poucos relatos sobre o aumento da atividade da acetilcolinesterase após exposição a agrotóxicos.

No presente estudo ocorreu lipoperoxidação no cérebro e músculo de carpas expostas em laboratório aos dois herbicidas comerciais, e também houve um aumento na formação de TBARS no tecido hepático das carpas expostas ao herbicida clomazone. Em condições de campo a lipoperoxidação foi observada em cérebro e fígado de peixes expostos ao herbicida clomazone. O aumento dos níveis de TBARS é um indicativo de estresse oxidativo, sendo reportado por outros autores. Crestani *et al.* (2007) encontraram um aumento dos níveis de TBARS no fígado e no cérebro de jundiás após exposição por 24 h ao herbicida clomazone (0,5 e 1,0 mg/L). Glusczak *et al*. (2007) também encontraram níveis elevados de TBARS em músculo de jundiás (*Rhamdia quelen*) expostos ao herbicida glifosato. Após 30 dias de exposição a ambos herbicidas, em condições de campo os níveis de TBARS foram aumentados no tecido muscular e diminuídos no cérebro dos peixes expostos ao clomazone. Moraes *et al*. (2007) observaram lipoperoxidação no tecido hepático e diminuição dos níveis de TBARS em músculo de piavas expostas ao herbicida clomazone por 30 dias, também em condição de campo. Neste estudo, aos 90 dias de exposição, a lipoperoxidação foi observada em cérebro e músculo de carpas após a exposição a ambas as formulações comerciais testadas.

Outro parâmetro importante em estudos que avaliam estresse oxidativo causado por contaminantes ambientais é atividade da enzima antioxidante catalase. Aos sete dias de exposição, em condição de laboratório, a enzima catalase mostrou sua atividade aumentada em fígado de carpas expostas a ambas as formulações comerciais, porém em campo a atividade da enzima não foi alterada. Quando esta enzima apresenta aumento de sua atividade, significa que está ocorrendo um processo de detoxificação, principalmente na tentativa de evitar ou minimizar a formação de TBARS nos tecidos. Monteiro *et al*. (2006) observaram resultados similares quando expôs *Brycon cephalus* ao inseticida metil paration (Folisuper 600). Entretanto, Crestani *et al.* (2007) observaram uma diminuição na atividade da enzima catalase em fígado de jundiás após exposição ao herbicida clomazone (0,5 e 1,0 mg/L). A diminuição encontrada na atividade da enzima catalase em jundiás foi relacionada com o aumento de TBARS encontrado no tecido hepático (CRESTANI *et al*., 2007). A enzima catalase não foi alterada após 30 dias de exposição aos herbicidas comerciais. Este resultado pode estar relacionado aos níveis de TBARS

hepático que também não sofreu alterações neste período de exposição. Porém após 90 dias de exposição ao herbicida clomazone, a atividade da enzima foi inibida em fígado de carpas. Após 21 dias da aplicação das formulações comerciais na lavoura, não foram encontrados resíduos destes herbicidas na água, porém alguns parâmetros permaneceram alterados nos tecidos de carpa.

A enzima glutationa S-transferase também tem um importante papel no sistema antioxidante, principalmente na detoxificação de xenobióticos. Aos sete dias de exposição a ambas as formulações comerciais, a atividade desta enzima não foi alterada. Porém, após 30 e 90 dias de exposição aos dois herbicidas comerciais, a atividade da enzima foi significativamente reduzida. Ballesteros *et al*. (2008) também encontrou uma diminuição na atividade da GST em fígado, músculo e brânquias de *Jenynsia multidentata* expostas ao inseticida endosulfan (0,014 a 1,4 µg/L). A carbonilação de proteínas também foi avaliada no presente estudo. Aos sete dias de exposição, ocorreu diminuição da proteína carbonil em fígado de carpas expostas ao herbicida clomazone. Porém após 30 e 90 dias de exposição a ambas as formulações comerciais, em condição de campo, observou-se um aumento na formação proteína carbonil no tecido hepático de carpas. A carbonilação de proteínas também é um indicativo de estresse oxidativo e tem sido usado como marcador de espécies reativas de oxigênio. Parvez & Raisuddin (2005), também observaram um aumento de carbonilação de proteínas em *Channa punctatus* expostos a diferentes pesticidas. Os resultados obtidos no presente estudo mostram que os elevados níveis de TBARS, juntamente com as alterações encontradas na atividade das enzimas catalase e glutationa S-transferase e o aumento da formação de grupos carbonila, indicam uma resposta adaptativa do peixe frente a possível toxicidade causada pelos herbicidas. Ocorreu dano oxidativo nos tecidos da carpa após a exposição a ambos herbicidas comerciais e períodos de exposição testados.

Após a exposição de carpas a ambas as formulações comerciais utilizadas, observaram-se desordens metabólicas, como alterações no conteúdo de glicogênio, lactato, glicose, proteína, amônia e aminoácidos. No geral, os resultados mostram que a redução de proteínas nos tecidos e no plasma provavelmente está relacionada ao consumo de proteína pelos tecidos a fim de obter energia para os processos metabólicos e de detoxificação do organismo. Esses resultados estão de acordo com o aumento da amônia observado após sete dias de exposição em ambos tecidos. O aumento da amônia pode ser devido ao catabolismo das proteínas para obtenção de

energia para os processos metabólicos. Porém após 30 e 90 dias de exposição a ambos herbicidas comerciais, os níveis de amônia foram diminuídos em fígado e músculo de carpas.

Diversos estudos que já avaliaram a exposição de peixes a pesticidas mostram uma redução no conteúdo de glicogênio (SANCHO *et al*., 1998; BEGUM & VIJAYARAGHAVAN, 1999; AGUIAR *et al*., 2004; FONSECA *et al*., 2008). Porém neste estudo, observou-se um aumento nos níveis de glicogênio hepático e muscular, após todos os períodos de exposição, nas duas condições experimentais e aos dois herbicidas comerciais testados. O aumento do glicogênio pode significar uma poupança dos carboidratos, e indica que o peixe nesta situação favorece a oxidação de proteínas. Um estudo realizado com jundiás também mostra um aumento de glicogênio hepático após 12-192 h de exposição ao herbicida clomazone (0,5 ou 1,0 mg/L) (CRESTANI *et al*., 2006). No presente estudo, o peixe reagiu ao estresse estocando energia na forma de glicogênio. Ao contrário da resposta observada para glicogênio e proteínas, os parâmetros glicose, lactato e aminoácidos mostraram resposta variada de acordo com o período e com o tecido considerado. Ocorreu aumento de glicose muscular e plasmática após exposição a ambos herbicidas comerciais em praticamente todos os períodos de exposição. Crestani *et al* (2006) também observaram os níveis de glicose plasmática aumentados em jundiás expostos ao herbicida clomazone. A glicose aumentada também serve como um indicativo secundário de estresse. Ocorreu variação dos níveis de lactato nos tecidos e plasma de carpas expostas a ambos herbicidas comerciais. O aumento de lactato indica que o músculo do peixe pode estar fermentando a glicose para obtenção de energia rapidamente. A diminuição do seu conteúdo pode indicar que está ocorrendo síntese de glicogênio e glicose, favorecendo assim a gliconeogênese. O aumento da amônia e a redução de proteínas no tecido muscular podem explicar a redução dos níveis de aminoácidos que ocorreu no músculo.

De uma maneira geral pode-se concluir que as duas formulações comerciais de herbicidas testados afetam os parâmetros indicadores de toxicidade em carpas. Em condição de laboratório, após sete dias de exposição, os efeitos em carpas parecem ser mais ofensivos que em campo. Porém, alguns efeitos permanecem após 90 dias de exposição, apesar dos herbicidas não serem mais detectados na água. Os parâmetros analisados podem servir de indicadores de toxicidade aos herbicidas estudados em carpas.

6 CONCLUSÕES

- As formulações comerciais dos herbicidas estudados provocam alterações na atividade da enzima acetilcolinesterase cerebral e muscular de carpas em ambas as condições experimentais. A formulação comercial do herbicida clomazone altera a atividade da enzima somente em condições de laboratório.

- A enzima catalase sofreu alterações que indicam um possível dano axidativo após exposição em laboratório. A enzima glutationa S-transferase apresentou somente alterações em campo, indicando uma resposta antioxidante. As alterações observadas na atividade destas enzimas em fígado de carpas representam um dano oxidativo causado pela exposição aos herbicidas comerciais testados.

- As modificações na formação de substâncias reativas ao ácido tiobarbitúrico (TBARS) em diferentes tecidos de carpas e o aumento dos níveis de carbonilação de proteínas no fígado deste peixe evidenciaram uma situação de estresse oxidativo. Estas formulações comerciais ocasionaram um desequilíbrio entre a atividade antioxidante e a formação de espécies reativas de oxigênio.

- Os intermediários metabólicos (glicogênio, proteína, glicose, lactato, amônia e aminoácidos) em fígado, músculo e plasma de carpas são alterados quando estes organismos são expostos a estas formulações comerciais. Os peixes utilizaram mecanismos compensatórios na tentativa de metabolizar os herbicidas.

- De acordo com os resultados obtidos após exposição em laboratório, pode-se afirmar que os efeitos foram mais agressivos que os obtidos em condições de campo.

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ANEXO

Anexo A

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMITÊ DE ÉTICA E BEM ESTAR ANIMAL

Emissão de Parecer
34/200 7

Nº Processo:	23081.010369/2007-58
Título:	Parâmetros toxicológicos em carpas(Cyprinus carpio var. húngara) expostos a formulações comercias de diferentes pesticidas em condições de lavoura de arroz e em laboratório
Pesquisador Responsável:	Profª. Vânia Lúcia Loro
Instituição:	UFSM
Área:	DEP. Química
Data de Entrada:	27/07/2007
Data do Parecer:	06.08.2007

Termo de compromisso com o bem estar animal:

( ) Termo de compromisso assinado pelo Coordenador e outros executores do projeto
(X) Termo de compromisso assinado pelo Coordenador se responsabilizando pelos demais executores do projeto
( ) Termo de compromisso assinado unicamente pelo Coordenador
( ) Termo de compromisso ausente no processo

Adequação e relevância do projeto:

(X) Problema científico relevante
(X) Hipótese adequada
(X) Objetivos relevantes
( ) Metodologia adequada

Caráter do projeto/ Linha de pesquisa quanto aos resultados esperados e benefícios potenciais, para a área em estudo e/ ou setores de aplicação da sociedade brasileira:

(X) Inovador / novo conceito
(X) Incremental (novas informações)
( ) Confirmatório
( ) Pouco relevante

Necessidade da utilização de animais na experimentação:

(X) O projeto necessita de animais para responder a pergunta científica
( ) O projeto não necessita de animais e pode ser realizado com metodologia in vitro ou similar

Análise do número de animais a ser utilizado no projeto:

(X) Adequado
( ) Inadequado
Base científica para classificar como inadequado e sugestão do número de animais que deverá ser utilizado:

Currículo do pesquisador em relação à área de abrangência do projeto:

(X) Adequado
( ) Inadequado

Avaliação global do projeto: 06/08/07

(X) Aprovado
( ) Aprovado com ressalva _____

( ) Reprovado (a proposta não está de acordo com a ética e bem estar animal)

Detalhe os pontos relevantes que o(a) levaram a avaliar negativamente o projeto:

Santa Maria, 06 de agosto de 2007.

Declaramos que o projeto Parâmetros toxicológicos em carpas(Cyprinus carpio var. húngara) expostos a formulações comercias de diferentes pesticidas em condições de lavoura de arroz e em laboratório registrado no Comitê de Ética e Bem Estar Animal sob número 23081.010369/2007-58, coordenado pelo pesquisador Vânia Lúcia Loro, cumpriu todas as exigências em relação ao Bem Estar Animal.

Tales Branda

Prof. Tales de Moura Branda
Presidente do Comitê de Ética e Bem Estar Animal

OBS: Entregar relatório ao final da execução/ao término do projeto