UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE JABOTICABAL
CENTRO DE AQÜICULTURA DA UNESP

RESPOSTA CORTISOLÊMICA E SENSIBILIDADE AO HORMÔNIO ADRENOCORTICOTRÓFICO (ACTH) DE JUNDIÁ (*RHAMDIA QUELEN*) EM EXPOSIÇÃO SUB-LETAL A AGROTÓXICOS

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Jaboticabal, SP
Maio 2009

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TÍTULO: RESPOSTA CORTISOLÊMICA E SENSIBILIDADE AO HORMÔNIO ADRENOCORTICOTRÓFICO (ACTH) DE JUNDIÁ (Rhamdia quelen) EM EXPOSIÇÃO SUB-LETAL A AGROTÓXICOS

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RESUMO

A intensificação das práticas agrícolas e o aumento da produção geralmente dependem da aplicação de agrotóxicos, que podem direta ou indiretamente contaminar as fontes de água, córregos, açudes e lençóis freáticos. O peixe jundiá (*Rhamdia quelen*) é uma espécie da família Siluridae que ocorre no sul da América do Sul. Devido a sua prolificidade, robustez e bom ganho de peso, a espécie tem sido intensivamente estudada. A resposta ao estresse é uma reação do organismo a uma variedade de fatores adversos e compreende uma série de processos fisiológicos adaptativos coordenados pelo eixo hipotálamo-hipófise-interrenal (eixo HHI). O impacto de contaminantes sobre a síntese dos hormônios corticosteróides ainda é pouco conhecido para peixes tropicais. Estes hormônios possuem importante papel em processos fisiológicos como crescimento, metabolismo, balanço hidromineral, reprodução e sistema imune. Assim, qualquer impacto no seu eixo neuroendócrino pode afetar o desempenho do animal. O efeito deletério dos contaminantes sobre o eixo HHI pode ser classificado como interrupção endócrina. Neste caso, um peixe sob estresse tem significativamente reduzida sua capacidade de elevar o cortisol plasmático e, assim, fica fisiologicamente comprometido e não responde adequadamente aos estressores comuns em seu ambiente. Assim, este trabalho avaliou a resposta de estresse de jundiás quando expostos á cinco agrotóxicos, por meio da avaliação da resposta cortisolêmica a estressor padrão e pelo teste de sensibilidade ao hormônio adrenocorticotrófico (ACTH). Com os resultados obtidos pode-se perceber que, a exposição sub-letal ao metil-paration, atrazine+simazine e glifosato exercem um efeito deletério na resposta cortisolêmica a um estressor adicional em jundiá (*Rhamdia quelen*) e que em teste de desafio ao ACTH, às concentrações sub-letais de tebuconazole e metil-paration (16% $CL_{50\text{-}96h}$) exercem um efeito sobre a capacidade da interrenal em elevar o

cortisol plasmático em resposta ao estímulo do ACTH, indicando que o tecido interrenal é o sítio do bloqueio no eixo HHI. Ainda, o teste do ACTH revelou uma diminuição da resposta cortisolêmica em peixes expostos ao atrazine+simazine e glifosato, indicando que a interrupção endócrina ocorre em outro nível do eixo HHI, que não sobre a interrenal.

1. INTRODUÇÃO

A contaminação dos recursos hídricos causada pelo uso de agrotóxicos nas lavouras adjacentes, tem recebido muita atenção devido aos impactos negativos causados tanto, sobre o ambiente natural, quanto sobre a produção de organismos aquáticos (Hori et al., 2008).

O estudo e a avaliação das exposições agudas a altas concentrações de agrotóxicos são facilitados pelo grande impacto e severa mortalidade que causam. Porém, as grandes maiorias das contaminações ocorrem com concentrações abaixo das consideradas letais que podem provocar um conjunto de alterações morfo-fisiológicas e metabólicas nos peixes (Barton et al., 1991).

É possível, ainda, estudar as alterações tóxicas por meio da avaliação de processos endócrinos e fisiológicos de peixes submetidos ou não aos agrotóxicos. Entretanto, pouco se sabe sobre o impacto destas contaminações sobre a síntese dos hormônios corticosteróides pelo tecido interrenal, homólogo da adrenal, e o grau de lesão causada nestas células, especialmente, em espécies de peixes tropicais. A elevação dos níveis de cortisol após estresse constitui-se na principal resposta adaptativa do organismo, pela ação chave nos processos de crescimento, metabolismo, balanço hidromineral, reprodução e função imune. Qualquer impacto no seu eixo neuroendócrino de controle pode potencialmente afetar o desempenho do animal (Hontela, 1998).

O conjunto de efeitos deletérios de *xenobióticos sobre a resposta ao estresse caracteriza-se como interrupção endócrina (Hontela, 1998). O peixe com a resposta de estresse diminuída tem sua capacidade de elevar o nível do cortisol plasmático significativamente prejudicado. Assim, fica fisiologicamente comprometido, podendo não responder adequadamente aos estressores comuns no ambiente natural ou de criação. A incapacidade de responder aos estressores torna o animal muito mais susceptível na manutenção de sua homeostase, o que, em nível populacional, pode diminuir a taxa de sobrevivência, o ganho de peso e refletir direta e negativamente no resultado econômico da atividade (Barcellos et al., 2006a).

Os xenobióticos capazes de provocar interrupção endócrina podem agir imitando ou bloqueando as ações dos hormônios, interagindo com seus receptores e mecanismos de ação e alterar a síntese do hormônio e/ou de seu receptor ou mesmo alterar a taxa de metabolização e/ou excreção do hormônio. Outra maneira dos agrotóxicos atuarem sobre a produção do cortisol pelo tecido interrenal é danificando as células que sintetizam este hormônio. Um dos danos celulares mais comumente relatado é o causado pelo estresse oxidativo.

* Xenobióticos: São compostos químicos estranhos a um organismo ou sistema biológico. O termo é também aplicado a substâncias presentes em concentrações muito mais elevadas que o nível normal. O organismo remove os xenobióticos através do denominado metabolismo ou desintoxicação de xenobióticos. Este consiste na neutralização e excreção destas substâncias; a neutralização ocorre principalmente no fígado e as principais vias de excreção são a urina, as fezes e a respiração. O grupo de enzimas do citocromo P450 é um dos envolvidos na desintoxicação de xenobióticos no fígado.

Além dos efeitos oxidativos sobre o sistema endócrino, outros processos fisiológicos podem ser afetados, como metabolismo energético e balanço hidromineral. Os peixes teleósteos são bons indicadores de contaminação por poluentes porque seu metabolismo responde de forma semelhante á outros teleósteos e a sua sensibilidade aos efeitos oxidativos pode ser igualmente medida (Dorval et al., 2005). Devido a isto, estudos que possam elucidar pontos relativos ao metabolismo de peixes em situações de contaminação ambiental, tornam-se indispensáveis para se ampliar o conhecimento básico dos efeitos deletérios dos toxicantes.

No sul do Brasil, os herbicidas glifosato, atrazine e Herbimix™ (combinação de atrazine + simazine) são utilizados em lavouras de soja e milho. Entretanto, esses agrotóxicos são considerados contaminantes aquáticos pelos efeitos que podem causar sobre a microbiota e ictiofauna local (Oulmi et al., 1995). O fungicida tebuconazole é muito utilizado em culturas de algodão (Lebokowska et al., 2003) e o inseticida metil-paration é utilizado em tanques para controlar as larvas de insetos predatórios dos peixes (Szarek et al., 2000).

O princípio ativo do Folicur 200CE, tebuconazole, rapidamente se degrada com baixa persistência no ambiente e não possui característica de bioacumulação (www.milenia.com.br). Já o methyl-paration (Folidol 600) é um inseticida organofosforado de baixa persistência, moderadamente solúvel em água e agudamente tóxico para os peixes (Walton et al., 1997). Atrazine e simazine são herbicidas pouco afetados pelos processos naturais de degradação e causam a contaminação quase permanente da superfície do solo e das água. As formulações do glifosato são rapidamente dissipadas na superfície das águas e biotransformadas pela microflora do solo e forma o

ácido α-amino-3-hydroxy-5-methyl-4-isoxazo-lepropionico (AMPA) e CO_2 (Glusczak et al., 2006).

Os agrotóxicos acima citados foram eleitos para a realização deste estudo devido à ampla utilização nas lavouras do estado do Rio Grande do Sul, onde o jundiá (*Rhamdia quelen*) também aparece com muita freqüência. A determinação das concentrações que foram utilizadas nos experimentos partiu do princípio de serem doses identificadas como não letais para a espécie (Kretuz et al., 2008), porém passíveis de causar algum efeito deletério sobre o eixo hipotálamo-hipófise-interrenal (HHI).

O conhecimento dos mecanismos de interrupção endócrina do sistema HHI é fundamental para que possamos tomar medidas preventivas sobre a presença de xenobióticos nos ambientes aquáticos, especialmente, nos sistemas de produção aquícola. Assim, com base no exposto, constata-se que os impactos negativos causados por concentrações sub-letais de agrotóxicos tornam-se indispensáveis, tanto na análise de contaminações ambientais, quanto para a produção sustentável e rentável de organismos aquáticos que ofereçam alto grau de segurança alimentar para os consumidores, melhorando a inserção e a qualidade sanitária dos peixes produzidos no Brasil.

2. REVISÃO DA LITERATURA

O jundiá (*Rhamdia quelen*), pertencente à família Siluridae, é uma espécie nativa da região sul da América do Sul (Barcellos et al., 2003) e considerada rústica, devido sua capacidade de suportar o intenso frio da região Sul do Brasil durante o inverno, bem como ter seu crescimento potencializado durante o verão (Soso et al., 2007). É um peixe onívoro de leve tendência carnívora, mas também se alimenta de plâncton maior e bento, tendo uma alta preferência por proteína de origem animal (Gomes et al., 2000). Este peixe é de fácil criação e possui boa aceitação pelo mercado consumidor, especialmente por ter uma carne branca, de sabor suave e possuir filés ausente de espinhas (Lazzari et al., 2006).

Devido à prolificidade, robustez e bom ganho de peso, o jundiá tem sido intensivamente pesquisado como alternativa de produção de peixes (Barcellos et al., 2004b; Silva et al., 2006) na avaliação de perfis hormonais reprodutivos (Barcellos et al., 2001b; 2002), resposta de estresse (Barcellos et al., 2001a; 2003; 2004a; 2006a e 2006b), efeito de agrotóxicos sobre a fisiologia (Kreutz et al., 2007), inclusive sobre a interrupção endócrina da reprodução (Soso et al., 2007). Por essas características zootécnicas e pela considerável quantidade de conhecimento é que a espécie foi escolhida como modelo experimental para o estudo da interrupção endócrina do eixo hipotálamo-hipófise-interrenal (HHI), provocada por contaminações químicas agudas.

A produção de peixes representa uma importante fonte de renda para o produtor rural e também a possibilidade de produzir e consumir um alimento saudável. No entanto, a construção e/ou localização da maioria dos açudes nos estados do sul do país ficam próximas a lavouras de produção de grãos ou utilizam água captada de bacia com lavouras

agrícolas. Com a intensificação das práticas agrícolas, a segurança da produção depende, muitas vezes, da aplicação de agrotóxicos, que podem direta ou indiretamente contaminar as fontes de água, córregos e açudes, e mesmo lençóis freáticos (Fioreze et al., 2006).

A contaminação direta resulta do despejo direto do produto nas águas, e comumente ocorre por falhas na aplicação (aplicações nas margens de rios e córregos) e principalmente, quando se utiliza aplicação aérea, com aviões agrícolas. Além disso, a contaminação das águas pode ser ocasionada pelo descarte de embalagens vazias não lavadas ou mesmo por produtos para o controle de plantas aquáticas, ou insetos predatórios (Fioreze et al., 2006).

A contaminação indireta ocorre quando o agrotóxico é aplicado de forma adequada, porém, é transportado até a rede hidrográfica pela água da chuva, ou por lixiviação. Quando em contato com a água, os produtos tóxicos podem entrar em contato com organismos aquáticos, intoxicá-los, e causar alterações significativas na biota local (Fioreze et al., 2006).

Normalmente, a contaminação da água por agrotóxicos está associada à mortalidade dos peixes, o que se constitui, geralmente, em uma intoxicação aguda, provocada por doses acima da sua concentração letal (CL_{50}). Este tipo de contaminação, geralmente, se constitui em um acidente ambiental. Entretanto, para a maioria dos produtos, a concentração do princípio ativo necessário para causar o efeito desejado nos organismos alvos é menor do que a concentração tóxica para animais terrestres e aquáticos e não impede a sobrevivência desses, mas causa alterações fisiológicas e bioquímicas dos organismos aquáticos (Kreutz et al., 2008).

Por não causar a morte imediata dos peixes, na maioria das vezes, este tipo de intoxicação dos peixes não é detectada. Segundo Kreutz et al. (2008) e Fioreze et al. (2006), uma das alternativas para o estudo da contaminação de águas com pequenas quantidades (sub-letais) de agrotóxicos é a determinação dos efeitos sobre as variáveis bioquímicas e fisiológicas que poderiam ser utilizados como indicadores da intoxicação, como os parâmetros sanguíneos, cortisol e o estresse oxidativo.

Durante o metabolismo de detoxificação dos xenobióticos pode ocorrer a formação de espécies reativas do oxigênio (EROS), que são substâncias oxidantes responsáveis por danos à parede celular e pela perda ou a diminuição das funções de um tecido ou órgão. Os danos induzido pelo EROS incluem alteração de macromoléculas celulares, tais como membrana lipídicas, DNA e/ou proteínas. Os danos podem alterar a função celular através de mudanças na absorção de cálcio ou pH intracelular e eventualmente pode levar a célula á morte (Swann et al., 1991). Estes eventos bioquímicos denominados de estresse oxidativo podem comprometer a função endócrina de uma glândula (Dorval et al., 2005).

Por outro lado, sistemas fisiológicos mais adaptados reagem aos efeitos do EROS pela reparação dos danos oxidativos ou pela eliminação dos radicais livres. Os sistemas anti-oxidantes, incluem enzimas anti-oxidantes (ex: superóxido dismutase, catalase, glutationa peroxidase e glutationa redutase) e eliminadores de radicais livres (ex: vitaminas C e E e glutationa), que removem o EROS, deste modo, protegendo os organismos do estresse oxidativo (Dorval et al., 2005).

Os estudos com vertebrados aquáticos e invertebrados têm demonstrado que o sistema anti-oxidante é um sensível indicador á exposição de xenobióticos (Livingstone et al., 1990 e Lemaire e Livingstone., 1993).

Em peixes, a modulação de sistemas anti-oxidantes no fígado pelo endosulsan, um organoclorado policíclico que interfere na função secretora de células esteridoigênicas da adrenal em teleósteos, e efeitos modulatórios da exposição ao cobre sobre a indução ao estresse oxidativo *in vivo* já foram reportados (Pandey et al., 2001). Estudos *in vitro* o endosulfan induz o estresse oxidativo em células da adrenocortical em truta arco-íris (*Oncorhynchus mykiss*) (Dorval et al., 2003).

Vários estudos *in vivo* e *in vitro* demonstram que alguns poluentes ambientais atuam como interruptores endócrinos em peixes (Leblond et al., 2001), e que um dos alvos dos xenobióticos é o eixo HHI (Hontela, 1998). Contudo, a interrupção endócrina e o potencial citotóxico de agrotóxicos como atrazine e endosulfan tem sido verificados *in vitro* nas células da interrenal de teleósteos e anfíbios (Leblond et al., 2001). Entretanto, não se sabe se exposições crônicas a campo á misturas de agrotóxicos, incluindo pesticidas induzem ao estresse oxidativo e causam dano à função da interrenal em teleósteos (Dorval et al., 2003).

Na produção de peixes, a ocorrência de estresse é praticamente inevitável, uma vez que, os peixes são submetidos a inúmeros manejos e a variação das condições ambientais, muitas vezes, desafiadoras. A resposta de estresse é uma reação do organismo a uma variedade de fatores adversos denominados de estressores que afetam uma série de processos fisiológicos coordenados pelo eixo hipotálamo-hipófise-interrenal (HHI). O

cortisol é o principal produto final deste eixo em peixes teleósteos e exerce uma variada gama de ações fisiológicas (Barton e Iwama, 1991 e Wendelaar Bonga, 1997). Por esta razão, a concentração do cortisol tem sido determinada no sangue de diversos peixes e utilizada como indicador para a avaliação da resposta de estresse a diversos estímulos.

A elevação dos níveis plasmáticos de cortisol em resposta a estressores é conhecida (Barton e Iwama, 1991), inclusive em jundiá (Barcellos et al., 2003, 2004a, 2004b, 2006 e 2009) e pode ser considerada uma resposta adaptativa, importante para os ajustes necessários para a manutenção da homeostase (Aluru e Vijayan, 2006).

Nos peixes, o tecido interrenal está distribuído no rim cefálico e é chamado de tecido interrenal. Assim como a adrenal dos mamíferos, este tecido é controlado pelo eixo hipotálamo-hipófise (Wendelaar Bonga, 1997). A síntese do cortisol no tecido interrenal envolve uma série de etapas iniciadas com a estimulação pelo hormônio adrenocorticotrófico (ACTH) e a conseqüente conversão do colesterol, através de uma série de passos enzimáticos, a esteróides (Payne e Hales, 2004).

O ACTH é um polipeptídeo com 39 aminoácidos produzido pelas células corticotróficas da adenohipófise. Atua sobre as células da interrenal, estimulando-as a sintetizar e liberar seus hormônios, principalmente o cortisol. A secreção de ACTH exibe ritmo circadiano com padrão diurno acentuado, controle por feedback negativo e respostas de ampla variedade de estímulos, entre eles o hormônio de liberação de corticotrofina. O ACTH tem como função estimular a secreção de hormônios da córtex supra-renal, principalmente glicocorticóides, além de manter a integridade da interrenal.

O controle da secreção do ACTH pode sofrer influência direta pela ação de fatores estressantes (densidade de estocagem elevada, confinamento, exposição á tóxicos...), pelo hormônio de liberação de corticotrofina (CRH) ou, ainda, pelo efeito do feed-back negativo de cortisol ao nível de hipotálamo e pituitária. Estes fatores, além de atuarem como moduladores na secreção do ACTH em peixes, refletem sobre a produção de cortisol (Lederis et al.1993).

Os níveis circulantes de ACTH, modulados pelo estresse, exercem um papel importante sobre a regulação do eixo HHI (Balm e Pottinger, 1995). Estes autores também discutem a hipótese de que a presença de altos níveis basais de cortisol liberado em peixes estressados seja devido a um estímulo crônico das células da interrenal e pela presença de altos níveis de ACTH circulante.

O tecido interrenal de peixes estressados é menos sensível ao estímulo do ACTH que de peixes não estressados (Mommsen et al., 1999). Em experimento com salmonídeos (*Salvenlinus fontinalis*) mantidos em estresse de alta densidade de estocagem, verificou-se uma diminuição na sensibilidade ao estímulo de ACTH, o que resultou em uma menor concentração de cortisol plasmático (Vijayan et al., 1998).

A dinâmica dos receptores de ACTH nas células da interrenal de peixes; entretanto, é provável que a regulação destes receptores desempenhe um papel importante na produção de cortisol associado ao estresse (Mommsen et al., 1999). Por exemplo, em testes *in vitro*, o inseticida organoclorado (β-NF) anulou completamente a resposta da produção de cortisol no tecido interrenal de peixes expostos ao tóxico, ainda que induzido pelo ACTH (Wilson et al., 1998). Esta resposta pode ser prejudicada pela ação de tóxicos

que diminuem a capacidade do tecido interrenal de produzir cortisol (Aluru e Vijayan, 2006).

Para Dorval et al. (2003) trutas arco-íris (*Oncorhynchus mykiss*) expostas ao inseticida endosulfam apresentaram aumento na peroxidação lipídica das células interrenais e alterações nas enzimas antioxidantes como a catalase, GSHpx (glutationa peroxidase) e GST (glutationa-S-tranferase). Estas alterações bioquímicas sejam responsáveis por danos celulares e, conseqüentemente, atuem na diminuição da produção de cortisol.

Assim, como os corticosteróides possuem importante papel em processos fisiológicos como crescimento, metabolismo, balanço hidromineral, reprodução e função imune, qualquer impacto no seu eixo neuroendócrino de controle pode afetar o desempenho do animal (Aluru et al., 2005). Assim, devido a esta grande relevância fisiológica, alterações neste eixo são consideradas importantes biomarcadores de poluição ambiental (Pacheco e Santos, 2001).

Diversos estudos sobre o impacto dos xenobióticos na esteroidogênese avaliaram a síntese de esteróides sexuais (Lal, 2007), inclusive em jundiá (Soso et al., 2007). O efeito sobre a resposta de estresse em diversas espécies (Gravel et al., 2005; Dorval et al. 2005), inclusive com demonstração do local de ação do xenobiótico em estudos *in vitro* (Lacroix e Hontela, 2003; Lizardo-Daudt et al., 2007). O efeito deletério dos xenobióticos na resposta de estresse pode ser classificado como interrupção endócrina.

A interrupção endócrina do eixo hipotálamo-hipófise-interrenal (HHI) tem sido associada à exposição crônica dos peixes aos xenobióticos em ambiente natural, como em *Perca flavescens* (Brodeur et al., 1997 e Gravel et al., 2005), *Catostomus commersoni* (Dorval et al., 2005) e mesmo em condições de laboratório em *Coregonus lavaretus* (Lappivaara, 2001). Isto por que, há pouco tempo atrás, acreditava-se que apenas a exposição prolongada aos tóxicos podia causar impacto neste eixo (Hontela, 1998; Jobling e Tyler, 2003 e Pottinger, 2003).

Para Pacheco e Santos (1996) a função interrenal pode também ser afetada adversamente por exposição aguda a ciclosfosfamida. A cortisolemia dos peixes expostos agudamente ao tóxico alvo do estudo estava baixa em relações aos controles não expostos, mas que o nível de cortisol no tecido interrenal destes peixes estava alto. Assim, propuseram que a interrupção endócrina ocorreu durante a liberação e não na síntese do hormônio. Segundo Pacheco e Santos (2001), o efeito de exposição aguda a xenobióticos derivados da destilação do petróleo em nível de secreção de cortisol pelas células interrenais e não apenas na liberação do hormônio, como verificado anteriormente e constataram a mesma diminuição do nível de cortisol em nível de interrenal.

Tanto as alterações endócrinas, quanto os danos oxidativos podem, paralelamente, afetar as células de outros tecidos como fígado e músculo e gerar impacto indireto sobre o metabolismo. O fígado é o tecido que metaboliza todas as drogas e responde primariamente a efeitos agudos causados por componentes tóxicos (Sancho et al., 1998). Entretanto, o músculo também tem o metabolismo alterado e mobiliza as reservas frente a um agente estressor, quer seja de natureza física ou química. Em piavas (*Leporinus*

friderici) expostas ao herbicida glifosato apresentaram aumento significativo do glicogênio hepático e redução do glicogênio muscular (Glusczak et al., 2006).

A redução do glicogênio no fígado e no músculo de peixes após a exposição aos agrotóxicos tem sido relatada em diversos estudos (Ghosh, 1987; Sancho et al.,1998; Aguiar et al., 2004). Entretanto, a resposta parece ser bem específica de alguns peixes que apresentam alterações nos níveis de glicogênio muscular como *Clarias batrachus* expostos a organofosforados (Begum e Vijayaraghavan.,1999). Porém, em outros este efeito não foi observado como *Anguilla anguilla* expostas a fenitrothion (Sancho et al., 1997).

Especificamente para o jundiá, *R. quelen*, Moraes et al. (2006) verificaram que a exposição ao herbicida glifosato reduziu a concentração do glicogênio hepático e muscular e aumentou os níveis de lactato, o que caracteriza uma situação de hipóxia tecidual, na qual os peixes adotaram a estratégia fermentativa para sobreviver. Alguns autores têm relatado também significativa redução da quantidade de proteínas em diferentes tecidos após a exposição agentes tóxicos (Sancho et al., 1998 e 2000; Glusczak et al., 2006).

Ainda que se encontrem estudos demonstrando os efeitos dos xenobióticos em peixes, são poucos os estudo com enfoque nas espécies tropicais e nativas do Brasil. Portanto, estudos adicionais são necessários para gerar novos conhecimentos sobre os efeitos destes agrotóxicos na fisiologia dos peixes, especialmente nas regiões onde a agricultura vem sendo desenvolvida com maior intensidade.

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a resposta cortisolêmica a um estressor padrão e resposta ao teste de sensibilidade ao hormônio adrenocorticotrófico (ACTH), em jundiá (*Rhamdia quelen*) expostos ou não a concentrações sub-letais de agrotóxicos.

3.2. Objetivos Específicos

3.2.1 Avaliar a resposta de cortisol a um estressor padrão em jundiá (*Rhamdia quelen*) expostos ou não a concentrações sub-letais de atrazine (Siptram500™), atrazine + simazine (Herbimix™), glifosato (Roundup™), metil-paration (Folidol600™) e ao tebuconazole (Folicur 200CE™).

3.2.2 Verificar, pelo teste de sensibilidade ao hormônio adrenocorticotrófico (ACTH), se os efeitos causados por estes agrotóxicos ocorrem por ação no tecido interrenal.

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Cortisol response to acute stress in jundiá *Rhamdia quelen* acutely exposed to sub-lethal concentrations of agrichemicals

ABSTRACT

Exposure to agrichemicals can have deleterious effects on fish, such as disruption of the hypothalamus–pituitary–inter-renal axis (HPI) that could impair the ability of fish to respond to stressors. In this study, fingerlings of the teleost jundiá (*Rhamdia quelen*) were used to investigate the effects of the commonly used agrichemicals on the fish response to stress. Five agrichemicals were tested: the fungicide (tebuconazole), the insecticide (methyl-parathion), and the herbicide (atrazine, atrazine+simazine, and glyphosate). Control fishes were not exposed to agrichemicals and standard stressors. In treatments 2–4, the fishes were exposed to sub-lethal concentrations (16.6%, 33.3%, and 50% of the LC50) of each agrichemical for 96 h, and at the end of this period, were subjected to an acute stress-handling stimulus by chasing them with a pen net. In treatments 5–7 (16.6%, 33.3%, and 50% of the LC50), the fishes were exposed to the same concentrations of the agrichemicals without stress stimulus. Treatment 8 consisted of jundiás not exposed to agrichemicals, but was subjected to an acute stress-handling stimulus. Jundiás exposed to methyl-parathion, atrazine+simazine, and glyphosate presented a decreased capacity in exhibiting an adequate response to cope with stress and in maintaining the homeostasis, with cortisol level lower than that in the control fish ($P<0.01$). In conclusion, the results of this study clearly demonstrate that the acute exposure to sub-lethal concentrations of methyl parathion, atrazine+simazine, and glyphosate exert a deleterious effect on the cortisol response to an additional acute stressor in the jundiá fingerlings.

1. INTRODUCTION

In southern Brazil, aquaculture is still considered as an activity complementary to agriculture, and thus, many ponds used for fish culture are located closer to or inside the agricultural areas, or are filled by water springs that run through the cultivated soil. Because of pest-management practices, large amounts of agrichemicals are used on crops and, as a result, small amounts of these products may reach the ponds used for fish culture (Van der Oost et al., 2003). In southern Brazil, the herbicides glyphosate, atrazine and Herbimix™ (a combination of simazine+atrazine) are widely used in soybean and corn cultures. However, these herbicides are considered as aquatic contaminants (Oulmi et al., 1995). The fungicide tebuconazole is used in plant cultures or as wood preservative (Lebokowska et al., 2003), and the pesticide methyl-parathion is used in fish-culture ponds to kill the aquatic larvae of the predatory insects (Szarek et al., 2000).

The active ingredient of Folicur 200CE, tebuconazole, rapidly degrades with short persistence in the environment, and is not bioaccumulative (http://www.milenia.com.br). However, methyl-parathion (Folidol 600) is a “less-persistent organophosphate insecticide, which is moderately soluble in water and acutely toxic to fishes (Walton et al., 1997). Atrazine and simazine are little affected by the natural degradation processes, resulting in almost permanent contamination of the surface and ground waters (Saglio and Trijasse, 1998). However, glyphosate formulations are rapidly dissipated from the surface waters, and undergo biodegradation by the soil microflora to form α-amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and CO2 (Glusczak et al., 2007).

Several field studies have focused on the prolonged exposures to agrichemicals, especially in wildlife, which adversely affect the hypothalamus pituitary–inter-renal axis (HPI) (Hontela, 1998). However, the possible effects on HPI axis caused by an acute exposure to these chemicals are not well described. Pacheco and Santos (1996, 2001) verified that fish acutely exposed to xenobiotics lost their capacity to elevate cortisol in response to additional stressors. Studies using experimental designs to access the HPI-endocrine disruption, caused by acute exposure to agrichemicals were not carried out for *Rhamdia quelen* or other fish that are suitable for production in the southern South America.

The HPI axis coordinates the stress response in fish, with adverse effects widely known (Wendelaar Bonga, 1997), including in jundiá (Barcellos et al., 2004). However, works focusing on the adaptive role of the stress response are rare for *R. quelen*. The end product of the HPI axis, the glucocorticoid cortisol, plays a key role in the metabolic and ionic adjustments necessary for coping with stress (Mommsen et al., 1999). Consequently, any adverse effect on the functioning of the HPI axis would compromise the ability of the animal to mount an adequate response to stressors (Hontela, 1998).

The jundiá (*R. quelen*), is an endemic species of southern South America, and is capable of growing in any region with a temperate or subtropical climate. Because of their characteristics, the jundiá has received great attention from the Brazilian researchers. Aspects of its reproductive physiology (Barcellos et al., 2002), larviculture (Townsend et al., 2003), stress response (Barcellos et al., 2004), toxicology (Kreutz et al., 2008), general physiology (Bello et al., 2000), and transportation (Golombieski et al., 2003) have already

been studied. Thus, *R. quelen* were the preferred models for our study, owing to their commercial importance, especially their well-characterized stress response.

Thus, the aim of this work was to verify if acute exposures to sub-lethal concentrations of the selected agrichemicals may affect the cortisol response to an acute stressor in *R. quelen* fingerlings.

2. MATERIALS AND METHODS

The experiments were conducted in March and April 2007, at the facilities of the Universidade de Passo Fundo, Rio Grande do Sul, Brazil. Were used 120-day old, mixed sex jundiá *R. quelen* (Heptapteridae, Teleostei) fingerlings, weighing 15.7±3.3 g (S.E.M., n=120). The fingerlings were kept in a 6200-L plastic tank prior to transferring into experimental tanks under natural photoperiod, and were fed twice a day (10:00 and 16:00 h) with commercial extruded food at 5% of body weight (42% crude protein, 3400 kcal kg^{-1} DE).

Water temperature (26±1 °C) and dissolved oxygen concentrations (5.6–7.5 mg L^{-1}) were measured with an YSI model 550A oxygen meter (Yellow Spring Instruments, USA). The pH values (6.6–7.0) (Bernauer pH meter), total ammonia-N (b0.5 mg L^{-1}) (colorimetric test), total alkalinity (60 mg L^{-1} $CaCO_3$), and hardness (65 mg L^{-1} $CaCO_3$) were also measured (colorimetric tests).

2.1. Experimental design and treatments

Five experiments were conducted, each one with specific agrichemical, tested with three sub-lethal concentrations (Table 1). The agrichemicals tested were tebuconazole (Folicur200CE™), methylparathion (Folidol600™), atrazine+simazine (Herbimix™), atrazine (Siptram500™), and glyphosate (Roundup™).

All the experiments consisted of 8 treatments with 4 replicates (total 32 tanks), containing 95-L chlorine free, well-aerated tap water and 10 fingerlings. Treatment 1 comprised the control (C) group, in which the fingerlings were kept in water without any agrichemicals and no stress was applied; in treatments 2, 3, and 4, the fingerlings were kept in water containing three sub-lethal concentrations of the agrichemical, corresponding to 16.6%, 33.3%, and 50% of the lethal concentrations for acute exposure (LC_{50}–96 h) (Kreutz et al., 2008), and after 95 h were subjected to an acute stress-handling stimulus (chasing them with a pen net for 60 s). In treatments 5, 6, and 7, the fingerlings were kept in water contaminated with the same sub-lethal concentrations for 96 h, but without any application of stress. In treatment 8, considered as the stressed (S) group, the fingerlings were kept in water without any agrichemicals, but were subjected to an acute stress handling stimulus after 96 h. The chemical name, commercial name, manufacturer, LC_{50}–96 h, and concentrations of the agrichemicals used in this study are listed in Table 1. In all treatments, the stocking density was 1.65 g L^{-1}, lower than the stocking density postulated as non stressful for *R. quelen* (2.2 g L^{-1}) (Barcellos et al., 2001a).

Table 1. Commercial and chemical name, manufacturer, usage, $LC_{50\text{-}96h}$ and the concentrations of agrichemical used.

Agrochemical	**Commercial name*** / manufacturer**	**Chemical name**	**Use**	**$LC_{50\text{-}96h}$* (mg L^{-1})**
Tebuconazole	Folicur™ Bayer SA	2-[2-(4-chlorophenyl)ethyl]-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)buta n-2-ol	Fungicide	5.3
Methyl-parathion	Folidol600™ Bayer SA	*O*-*O*-dimethyl *O*-4-nitrophenyl thiophosphate	Insecticide	4.8
Atrazine+Simazine	Herbimix™ Milenia SA	6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine + 2-chloro-4-ethylamine-6-isopropylamino-S-triazine	Herbicide	10.5
Atrazine	Siptram500™ SipCam Agro	6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine	Herbicide	10.2
Glyphosate	Roundup™	N-phosphonomethylglycine	Herbicide	7.3

*(Kreutz et al., 2007); ** refers to percentage of the $LC50_{\text{-}96h}$. ***Commercial names might be trademark protected by law. All products were purchased on local stores.

The five experiments were carried out in a static-test design. Usually, in such experiments, fishes are not fed; however, since cortisol is a glucocorticoid that might be affected by starvation, the fishes in this study were fed thrice during the 96 h of exposure (24, 48, and 72 h after the beginning of exposure) at a rate of 0.75% of their biomass. Food residues and feces were not removed, to prevent stress owing to the introduction of cleaning equipment. The water quality was monitored daily.

Acute stress-handling stimulus was applied after 96 h of the experiment. One hour after the stress application, all the fishes were sampled, since earlier results indicated that cortisol peaks in *R. quelen* occur after 1 h following the stress application (Barcellos et al., 2001a).

2.2. Blood sampling

Fish were anesthetized by administering buffered (NaH_2CO_3) MS222 (300 mg L^{-1}) into the tank. After loss of orientation and complete immobilization, the fishes were captured and blood samples (0.1–0.30 mL) were taken from severed caudal peduncle, using sterile microhematocrit tubes. The time elapsed from anesthesia to blood collection did not exceed 1 min. Tubes were centrifuged (3000-g, 10 min) in a microhematocrit centrifuge, and the plasma was collected using a Hamilton syringe, transferred to 1.5 mL microcentrifuge tubes, and stored at −25 °C.

After blood collection, the fishes were killed by spinal section and decapitation.

2.3. Hormone measurement

The cortisol was measured in duplicates, in the unextracted plasma samples, using commercially available EIA kits (EIAgenTM Cortisol, Adaltis Italy S.p.A). The specificity of the test was evaluated by comparing the parallelism between the standard

curve and serial dilutions in PBS (pH 7.4) of the plasma samples. The standard curve, constructed with human standards ran parallel to that obtained using serial dilutions of jundiá plasma. In the linear-regression test, a high positive correlation (R_2=0.98) was observed between the curves. The inter- and intra-assay coefficients of variation ranged from 9% to 12% and 6% to 9%, respectively.

2.4. Statistics

The mean ± S.E.M. (n=4) of each group was calculated and analyzed with Graph Pad InStat 3.00 statistical package (GraphPad Software, San Diego, CA, USA). Cortisol values of all treatment groups were compared using a two-way analysis of variance (ANOVA) to determine the effect of the treatment variable (agrichemical exposure) on the stress variable (cortisol level), and the significant differences were tested using the Student–Newman–Keuls test (Zar, 1996); cortisol levels of all the fishes of the control group (treatment 1) and the stressed group (treatment 8) were compared by ANOVA and the significant differences were tested using Tukey's test, with a significance level of 0.05. A Hartley test was carried out to verify the homogeneity of variance, and normality was tested using Kolmogorov–Smirnov test. Log transformation was performed when necessary, while non-transformed data are shown in the figures.

3. RESULTS

3.1. Cortisol measurements

3.1.1. Cortisol level of the control group

The cortisol levels found in the control fingerlings varied from 17.5±7.92 to 38±13.59 ng mL^{-1}, and were similar with no statistical differences (Fig. 2A, B, C, D, and E; P=0.30).

3.1.2. Cortisol level in the stress group

In all the experiments, fingerlings kept in agrichemical-free water, but subjected to handling stress, had a marked increase in the plasma cortisol levels, varying from 126.8±35.8 to 212.8±14.6 ng mL^{-1}, which were statistically similar (P=0.15).

3.1.3. Experiment with tebuconazole

Cortisol level in fishes exposed to tebuconazole in treatments 5, 6, and 7 (Fig. 2A) was similar to that in the control fingerlings, indicating that the sub-lethal concentrations of this agrichemical did not cause stress. In addition, tebuconazole did not block jundiá fingerlings to respond to an additional acute stressor in treatments 2, 3, and 4 (Fig.2A).

3.1.4. Experiment with methyl-parathion

Fingerlings exposed to methyl-parathion had cortisol levels similar to those of the control fingerlings. However, the presence of methyl-parathion indicates suppression on the fingerlings' ability to respond to additional stress (Fig. 2B).

3.1.5. Experiments with atrazine and atrazine+simazine

Sub-lethal concentrations of atrazine+simazine or atrazine alone induced a raise in the cortisol level in the jundiá fingerlings (Fig. 2C and D), suggesting that these products may be stressful *per se*. The response to an additional acute stress in fingerlings subjected to atrazine+simazine or atrazine alone could not be determined, as both the products induced a cortisol response by themselves (Fig. 2C and D).

3.1.6. Experiment with glyphosate

The presence of three sub-lethal concentrations of glyphosate provoked a slight increase in the cortisol level in fingerlings exposed to it, which was lower than the value verified in the stressed fishes (Pb0.01), but higher than that of the control ones (Pb0.01). The highest concentration (50% of $LC_{50–96\ h}$) of the agrichemical attenuated the cortisol response to an acute additional stressor, with the cortisol values lower than those found in the lower concentrations (Pb0.01).

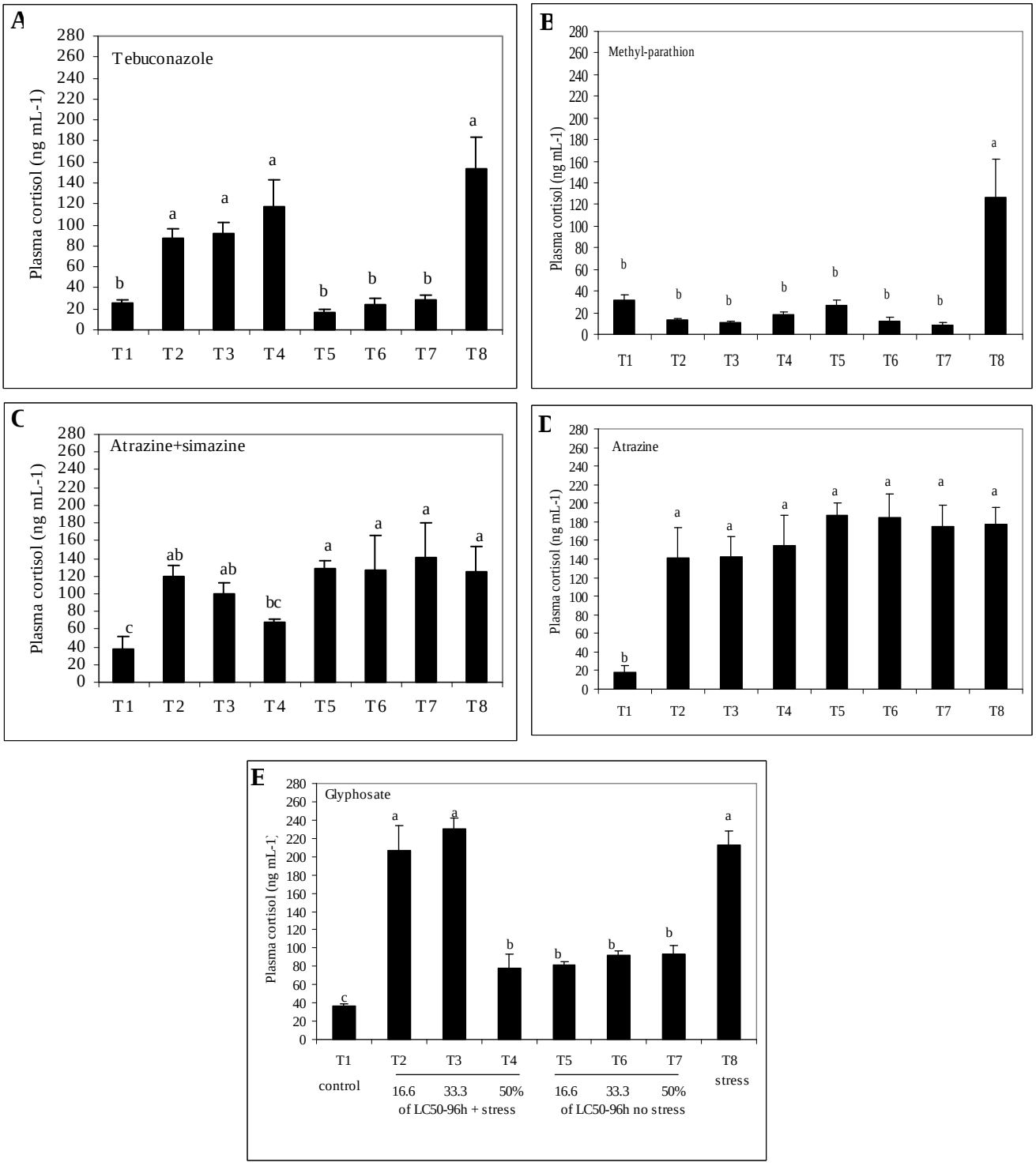

Figure 2. Plasma cortisol levels (mean ± SEM) in *Rhamdia quelen* submitted or not to acute stress after acute exposure (96h) to tebuconazole, methyl-parathion, atrazine+simazine, atrazine and glyphosate. T1: control treatment; T2, T3 and T4: fish submitted to handling stress after 96 hours of exposure to agrochemicals. T5, T6 and T7: fish exposed to 16.6, 33.3 and 50% of $LC_{50\text{-}96h}$ of each agrochemical, respectively. T8: fish not exposed to agrochemical, but submitted to stress. Different letters above bars indicates statistical differences between different treatments. Two-way ANOVA and SNK test, ($P<0.05$).

4. DISCUSSION

4.1. Water quality

All water-quality parameters measured during the experiments were within the optimal range (Boyd, 1982), indicating that the feeding regime had no effect on the water quality, probably due to the small stocking density and low food-rates.

4.2. Cortisol levels in control fish

The absence of deleterious effects induced by feedings strengthened by the cortisol concentrations measured in the control fingerlings, which were similar to those determined previously (Barcellos et al., 2004) in *R. quelen* fingerlings of similar age and size.

4.3. Cortisol levels in stressed fish

Despite the variations observed in the cortisol levels of stressed fish in all the experiments, statistically, the data were similar, suggesting that the experimental stressor was effective to cause a typical stress response, consistent with the responses previously measured in *R. quelen* (Barcellos et al., 2001a, 2003, 2004, 2006a,b).

4.4. Tebuconazole

The presence of tebuconazole in sub-lethal concentrations did not cause elevation or affect the cortisol response to an additional stressor. Data on the effects of tebuconazole on fish are inadequate, and often focused on the behavioral changes (Hussar et al., 2004). In this study, *R. quelen* fingerlings exposed to tebuconazole had no behavioral alterations. However, the possibility that tebuconazole might cause deleterious effects on the cortisol response in chronic or sub-chronic exposures should be further evaluated.

4.5. Methyl-parathion

The most important observation on fishes sub-lethal exposed to methyl-parathion in all the concentrations tested is their total impairment to elevate cortisol in response to acute stress.

The organophosphorus methyl-parathion, commonly known as Folidol600®, with commercial formulae containing 600 g L^{-1} of the active substance, is widely used as an insecticide in food storage and agriculture, as well as in fish farms to eliminate predatory aquatic insects and monogynies. Thus, the current uses and practices easily allow the possibility of methyl-parathion to reach non-target organisms like fishes (Fanta et al., 2003), impairing their ability to cope with stress. This observation is strengthened by the data obtained from jundiás exposed to sub-lethal methyl-parathion concentrations and subjected to an acute stressor. This effect can be classified as the endocrine disruption of the HPI axis. An attenuated cortisol response in fishes subjected to acute stress in the presence of untreated bleached kraft-mill effluent (BKME) has also been observed earlier (Lappivaara, 2001), and the involvement of altered hepatic-enzyme cascades in the

attenuation of the stress response has been suggested. Nonetheless, a weakened cortisol response to acute stress is a well-documented phenomenon that occurs in several fish species, following prolonged exposures to xenobiotics (Hontela et al., 1997; Girard et al., 1998; Norris et al., 1999; Dorval et al., 2005). However, data about the diminished cortisol response following an acute exposure are limited (Pacheco and Santos, 2001; Gravel and Vijayan, 2007; Hori et al., 2008). A possible physiological mechanism behind this blocked cortisol response seems to be related to the decreased responsiveness of the inter-renal tissue to the adrenocorticotropic hormone (ACTH), as verified by Benguira et al. (2002). They demonstrated that rainbow trouts, subjected to a single injection of o.p′-DDD (o.p′-dichlorodiphenyldichloroethane) lost their capacity to elevate plasma cortisol after confinement stress, in seven days post-injection.

Methyl-parathion or its more toxic-metabolite, methyl-paroxon (Loomis and Hayes, 1996), which is slowly inactivated in fishes than in mammals (Areechon and Plumb, 1990), might exert an adrenotoxicity, provoking impairment of the cortisol response. This could explain the block in cortisol response, 4 days after the exposure to sub-lethal concentrations of methyl-parathion. In accordance to this possible cellular effect of methyl-parathion, Pickering (1981) postulated that the first effects of contaminants usually occur at the cellular level. Confirming this assumption, methyl-parathion caused histopathological abnormalities, as soon as 1 h after the contamination in *Corydoras paleatus* (Fanta et al., 2003). Abnormalities at the cellular level caused by methyl parathion were also verified in *Mystus cavasius* (Murphy et al., 1984).

Further evidence of HPI impairment after acute exposure to agrichemicals is provided by Gravel and Vijayan (2007), who observed inhibited adaptive plasma-cortisol

response and the associated metabolic hanges in the liver of trouts fed with salicylate-laced feed for only 3 days. They postulated that the mode of action of salicylate involves disruption of StAR and liver GR, the two key proteins critical for the cortisol production and target-tissue responsiveness to this steroid, respectively. These hypotheses were also postulated by Hori et al. (2008) to explain the HPI blockage in matrinxã (*Brycon amazonicus*) after acute exposure (96 h) to low concentrations of phenol.

Recently, the oxidative stress in the inter-renal cells was suggested as a possible cause of endocrine disruption (Dorval et al., 2005). Since, methyl-parathion has the potential to induce oxidative stress in fishes (Monteiro et al., 2006), in the kidneys (Singh et al., 2006), the possible effects related to the oxidative stress in the mechanism of impairment of cortisol response in *R. quelen* should be considered.

4.6. Atrazine+simazine and atrazine

The combination of atrazine+simazine (Herbimix®) in sub-lethal concentrations caused a significant elevation in the cortisol levels, similar to those measured in acutely stressed fishes without Herbimix®, and jundiá acutely stressed in the presence of lower Herbimix® concentrations (16.6% and 33.3% of $LC_{50\ -\ 96\ h}$). However, at the highest concentration (50% of $LC_{50\ -\ 96\ h}$), the cortisol response seems to be attenuated, with values similar to that in the control fishes. This pattern indicates that Herbimix caused cortisol elevation, but did not block the cortisol response at lower concentrations. A similar pattern was verified in fish acutely exposed to atrazine alone, except that no attenuation of cortisol response was observed with the concentrations tested.

A possible explanation for the increased level of cortisol in fishes exposed to Herbimix® and atrazine alone, without standard stressor, is given by Prassad and Reddy (1994) who demonstrated a clear negative effect of atrazine on the hydromineral balance of *Oreochromis mossambicus*. The altered hydromineral balance was also verified by Waring and Moore (2004) in *Salmo salar* smolts, in which atrazine provoked a marked response of cortisol. The same pattern was also observed by Nieves-Puigdoller et al. (2007) in Atlantic salmons. Similarly, Fortin et al. (2008) concluded that short-term exposure to atrazine affects the osmotic control in mummichog (*Fundulus heteroclitus*). The role of cortisol on hydromineral balance is well known (Wendelaar Bonga, 1997) and thus, the osmo-regulatory dysfunction provoked by atrazine, mainly the increase in osmotic water-influx, might have caused a cortisol elevation, aiming to restore the hydromineral balance. This osmo-regulatory dysfunction might be harmful per se, and owing to sustained high cortisol level, it may cause several deleterious physiological changes, affecting the immunocompetence, the health and survival of the fishes (Wendelaar Bonga, 1997).

Interestingly, despite the fact that Herbimix® causes cortisol elevation, the exposure of *R. quelen* fingerlings to 50% of $LC_{50–96\ h}$ of this herbicide for 96 h caused attenuation of the cortisol response to the additional acute stressor. Since fishes exposed to the same concentrations of atrazine did not show this attenuation, the effect seems to be more related to the presence of simazine, or as a result of the combination of both the active substances. While triazine herbicides may cause liver and kidney damages, the mechanism involved in the attenuation of cortisol response to stress, provoked by simazine might be related to adrenotoxicity. The dose-dependent effect is consistent with the moderate histopathological changes related to this herbicide group. However, the

attenuation of cortisol response could be verified only at higher concentrations of the combination of atrazine+simazine.

Atrazine caused liver and kidney damage and altered the liver enzymatic pathways in common carp (Neskovic et al., 1993), but these alterations were considered as a moderate pathological response, as the function of the organ was not seriously affected. These could explain the fact that the exposure to atrazine did not block the cortisol response to additional acute stress in fishes in this study. In contrast, adrenotoxic effects of atrazine were observed in the amphibian species (Goulet and Hontela, 2003) and in rainbow trouts (Bisson and Hontela, 2002).

4.7. Glyphosate

Cortisol levels in fish exposed to the three sub-lethal concentrations of glyphosate were higher than control levels, indicating that this compound (or the surfactant associated in its commercial formulation) provoked stress in *R. quelen* juveniles. The toxicity of glyphosate is considered to be low, but the commercial glyphosate formulations, such as the Roundup™, are more acutely toxic than glyphosate, owing to the presence of surfactants like polyoxyethyleneamine (POEA), which is more toxic to fishes than glyphosate (Glusczak et al., 2007). The stress-inducing potential of Roundup™ for *R. quelen* confirmed the observations by Soso et al. (2007), who demonstrated that the endocrine-disrupting effect of glyphosate in *R. quelen* was targeted to the hypothalamus–pituitary–gonadal axis. Furthermore, in *R. quelen*, the oxidative stress caused by glyphosate was observed in the kidney (Glusczak et al., 2007). The glyphosate-based

herbicide also induced oxidative damage in some tissues of the neotropical fish, *Prochilodus lineatus* (Langiano and Martinez, 2008).

Despite the exact mechanism involved in the cortisol-response attenuation caused by methyl-parathion, atrazine+simazine, and glyphosate, biologically, the fishes lost their capacity to mount an adequate response to cope with a standard stressor and maintain homeostasis. This attenuation may reduce the adaptive response and the ability of the organism to promote metabolic and ionic adjustments necessary in the stress response. As reviewed by Brodeur et al. (1997), fishes incapable of mounting a normal cortisol response are likely to have a reduced ability to respond to the continuous challenges imposed on their homeostatic systems, either by aquaculture practices or by the environmental changes.

In conclusion, our results clearly demonstrate that sub-lethal exposure to methyl-parathion and atrazine+simazine exerted a deleterious effect on the cortisol response to an additional acute stressor in *R. quelen* fingerlings. This impairment of cortisol response may seriously hamper the adaptive response, as well as the ability to promote metabolic and ionic adjustments necessary to cope with stress.

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Responsiveness of the interrenal tissue of Jundiá (*Rhamdia quelen*) to an in vivo ACTH test following acute exposure to sub-lethal concentrations of agrichemicals

ABSTRACT

As in many aquatic environments, water pollution is a widespread problem in Southern Brazil. In our previous work, we demonstrated that sub-lethal contamination with some agrichemicals impairs the capacity of fishes to elevate cortisol levels in response to an additional acute stressor. In the present work, we used the adrenocorticotropic hormone (ACTH) challenge test to help us identify if the impairment occur in the interrenal tissue. For this purpose, five experiments were conducted, each with one specific agrichemical (methyl-parathion, atrazine + simazine, atrazine, tebuconazole, and glyphosate) in sub-lethal concentrations of 16.6% of the $LC_{50\ 96h}$, as previously determined. The fishes were subjected to the ACTH challenge test protocol as follows: group 1, were non-injected and maintained as the specific control group; group 2 received an injection of the vehicle alone (the saline group); and group 3 receive an injection of ACTH. One hour later, blood samples were taken from the caudal plexus, using sterile syringes. In all specific control groups, the injection of ACTH induced a strong rise in plasma cortisol, compared with the fishes injected only with the vehicle and the non-injected group. Fishes exposed to methyl-parathion and tebuconazole did not elevate cortisol in response to the ACTH injection, with values significantly lower than the control fishes. Fishes exposed to sub-lethal concentrations of atrazine + simazine, atrazine, and glyphosate showed a rise in plasma cortisol very similar to the control fishes. The conclusion is that the ACTH challenge test revealed that *R. quelen* exposed to sub-lethal concentrations of tebuconazole and methyl-parathion had a reduced ability to elevate plasma cortisol in response to an intraperitoneal (i.p.) injection of exogenous ACTH, indicating that the interrenal tissue is the site of the

impairment within the HPI axis. These ACTH challenge tests also revealed that the impairment of the cortisol response verified in fishes exposed to atrazine + simazine and glyphosate, as shown in our previous work, seems to be related to steps of cortisol secretion in higher levels within the HPI axis.

1. INTRODUCTION

Water pollution is a widespread problem in many aquatic environments (Hori et al., 2008). In Southern Brazil, water pollution occurs because most ponds used for fish culture are located close to or within agricultural areas, or are filled by water springs that run through cultivated soil. Modern crop production uses large amounts of agrichemicals, and small amounts of these products may reach ponds used for fishes culture (Van der Oost et al., 2003).

The herbicides glyphosate, atrazine, and Herbimix^{TM} (a combination of simazine and atrazine), which are widely used in Southern Brazil, are considered aquatic contaminants (Oulmi et al., 1995). The fungicide tebuconazole is used in plant cultures or as a wood preservative (Lebokowska et al., 2003) and the pesticide methyl-parathion is used in fishes culture ponds to kill the aquatic larval stages of predatory insects (Szarek et al., 2000). Tebuconazole, the active ingredient of Folicur 200 CE, degrades rapidly, with short persistence in the environment, and is not bio-accumulative (*Milenia, 2008*). Methyl-parathion (Folidol 600) is an organophosphate insecticide with short persistence in environment that is moderately soluble in water and acutely toxic to fishes (Walton et al., 1997). Atrazine and simazine are affected minimally by natural degradation processes, resulting in almost permanent contamination of surface and ground waters (Saglio and Trijasse, 1998). However, glyphosate formulations rapidly dissipate from surface water, and undergo biodegradation by the soil microflora to form α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and CO_2 (Glusczak et al., 2007).

The cortisol is the main glucocorticosteroid secreted by the interrenal tissue of teleosts in response to stimulation by the adrenocorticotropic hormone (ACTH). Cortisol levels rise when an organism is subjected to stressors (Barton and Iwama, 1991). The activation of the hypothalamus-pituitary-interrenal (HPI) axis and the increased plasma cortisol enable the fishes to mobilize energy substrates to cope with stressors. Due to this significant role of cortisol in homeostatic mechanisms, the impairment of the cortisol stress response, diagnosed in fishes exposed to pollutants, may adversely affect fish health.

In previous work (Cericato et al., 2008), demonstrated that sub-lethal contamination with some agrichemicals impairs the capacity of fishes to elevate cortisol in response to an additional acute stressor. The experimental design of those studies did not allow us to conclude where this effect occurs within the HPI axis. In the present work we used the ACTH challenge test to help us to identify if the site of the impairment was the interrenal tissue (as reviewed by Girard et al., 1998). The ACTH challenge test was used to assess the capability of the interrenal tissue to secrete cortisol in fishes exposed to pollutants in field and laboratory conditions (Hontela, 1998; Girard et al., 1998).

Jundiá (*R. quelen*) is an endemic species of southern South America, and is capable of breeding in any region with a temperate or subtropical climate, receiving great attention from Brazilian researchers (Gomes et al., 2000; Barcellos et al., 2004). *R. quelen* was the preferred model for our study because of its commercial importance, and especially due to its well characterized stress response (Barcellos et al., 2004, 2006a,b) and due previous studies focusing toxicological aspects (Soso et al., 2007; Kreutz et al., 2008)

The aim of this study was to investigate the response of *R. quelen* to an ACTH challenge test after acute exposure to sub-lethal concentrations of selected agrichemicals, and to verify whether the endocrine-disrupting effects (Cericato et al., 2008) were exerted in the interrenal tissue.

2. MATERIALS AND METHODS

The experiments were conducted from April to June 2008, at the facilities of the Universidade de Passo Fundo, Rio Grande do Sul, Brazil. We used 6-month-old mixed-sex *R. quelen* juveniles weighing 115.7 ± 23.3 g. Prior to distribution into the experimental tanks, the fishes were kept in a 6200-L plastic tank, under natural photoperiod, and fed commercial extruded food (42% crude protein, 3,400 Kcal Kg^{-1} DE) twice a day (at 10:00 and 16:00 h), receiving 5% of body weight per feed. The water exchange rate was 10% per day.

Water temperature (26º ± 1º C) and dissolved oxygen concentrations (5.6 to 7.5 mg L^{-1}) were measured with an YSI model 550A oxygen meter (Yellow Spring Instruments, USA). Also measured were pH values (6.6 to 7.0, using a Bernauer pH meter), total ammonia-N (less than 0.5 mg L^{-1}), total alkalinity (60 mg L^{-1} $CaCO_3$), and hardness (65 mg L^{-1} $CaCO_3$), all using a colorimetric tests.

2.1. Experimental design and treatments

Five experiments were conducted, each with one specific agrichemical, tested in a sub-lethal concentration of 16.6% of the $LC_{50\text{-}96h}$, as previously determined by Kreutz et al. (2008). The agrichemicals tested were methyl-parathion (Folidol 600™), atrazine + simazine (Herbimix™), atrazine (Siptram 500™), tebuconazole (Folicur 200 CE™), and glyphosate (Roundup™).

Each experiment consisted of groups of *R. quelen* in six fiberglass tanks containing 1000-L chlorine-free, well-aerated tap water. The fish in the tanks 1 to 3 did not receive any chemical contamination and were considered the general control group. The water in the other three tanks was artificially contaminated with 16.6% of the $LC_{50\text{-}96h}$ of the specific agrichemical tested. Three net enclosures were installed in each tank, and fishes were kept at a density of five fishes per enclosure. In all the six tanks, the stocking density was 2.3 mg L^{-1}, similar to the stocking density postulated as non-stressful for *R. quelen* (Barcellos et al., 2001).

After 96 h of exposure (or not), all the fish were subjected to the ACTH challenge test protocol as follows: the fish in enclosures 1 were non-injected (non-injected group); the fish in enclosures 2 received an injection of the vehicle alone (saline group), and the fish in enclosures 3 received an injection of porcine $ACTH^{1\text{-}39}$ (ACTH group) as described later.

The experiments were carried out in a static test design. Generally, fish are not fed in such experiments, but as cortisol is a glucocorticoid that might be affected by starvation, the fish of the present study were fed three times during the 96 h of exposure (at 24, 48, and 72 h after start of exposure to the agrochemicals) at a rate of 0.75% of biomass. To prevent stress due to the introduction of cleaning equipment, food residues and feces were not removed. The water quality was monitored daily by determining DO, pH, and total ammonia.

After each experiment, the contaminated water was kept for at least 30 days in fiberglass tanks and then percolated in septic ponds. After each experiment, tanks were cleaned with running water followed by rinsing with ethanol. Before reuse, tanks were filled with water and tested for remaining toxicity by adding *R. quelen* fingerlings that were observed for at least 5 days for mortality or behavioral changes.

2.2. Preparation and use of ACTH

Immediately before taking the fishes out of the enclosures, porcine $ACTH^{1-39}$ (Sigma Corp., St. Louis, MO.) was dissolved in saline (0.7% NaCl), transferred into a 1-mL syringe, and kept on ice. The ACTH group received an i.p. injection of 2 IU ACTH $100g^{-1}$ body mass (BM) per 100 μL of saline, and the saline group received an i.p. injection of 100 μL saline 100^{-1} g BM. The third group did not receive any injection. After injection, the fishes were put back in the enclosures and were sampled 1 h later, using the protocol described by Barcellos et al. (2006a).

The ACTH dose and the length of time before sampling (1 h) were selected in a pilot study and based on the data of Barcellos et al. (2001; 2003; 2004; and 2006a,b) pertaining to the *R. quelen* stress response. The pilot study was conducted in the same laboratory conditions, and the *R. quelen* were injected with saline or with three different doses of ACTH (1, 2, or 4 IU). The doses of 2 and 4 IU ACTH elicited a significantly higher cortisol response (257.8 ± 9.6 ng mL^{-1} and 261.78 ± □26.89 ng mL^{-1}) than the dose of 1 IU (153.8 ± 0.12 ng mL^{-1}), and the cortisol level in fishes injected with 2 and 4 IU was higher than in fishes injected with saline.

2.3. Blood sampling

For blood sampling, the fish were captured and anesthetized with buffered ($NaHCO_3$, 600 mg L^{-1}) MS222 (Finquel ®, 300 mg L^{-1}). After loss of orientation and complete immobilization, the fishes were captured and blood samples (1-2 mL) were taken from the caudal plexus, using sterile syringes. The time that elapsed from the time of administering anesthesia to the time of blood collection was less than 1 min. The blood samples were transferred to Eppendorf tubes and then centrifuged (3000-g, 10 min), and the plasma was stored at -25^{o} C until analysis.

After blood collection, the fishes were killed by spinal section and decapitation. All the dead fishes were frozen and subsequently shipped to the biological garbage collector.

2.4. Hormone measurement

The cortisol was measured in duplicate, in unextracted plasma samples, using commercially available EIA kits (EIAgen^{TM} Cortisol, Adaltis Italy S.p.A). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions of the plasma samples in phosphate buffered saline (pH 7.4). The standard curve constructed with the human standards ran parallel to that obtained using serial dilutions of *R. quelen* plasma. In the linear regression test, high positive correlation (R^2 = 0.9818) was found between the curves. The inter- and intra-assay coefficients of variation ranged from 9 to 12% and from 6 to 9%, respectively.

2.5. Statistics

The mean ± S.E.M. of each group was calculated and analyzed using the Graph Pad InStat 3.00 statistical package (GraphPad Software, San Diego, California, USA). The cortisol values of all the treatment groups (ACTH, saline and non-injected) were compared by two-way analysis of variance (ANOVA), followed by Dunnett's tests to compare each value against the control value (non-injected group in water not contaminated). Statistical significance was accepted at $p<0.05$. A Hartley test was carried out to verify the homogeneity of variance, and normality was tested using Kolmogorov–Smirnov test. Log-transformation was performed when necessary, while non-transformed data are shown in the figures.

3. RESULTS

3.1. Cortisol response pattern (Figure 1)

3.1.1. Control groups

The injection of ACTH in the control groups induced a strong rise in plasma cortisol ($p<0.001$), compared with fishes injected only with the vehicle (saline group), and the non-injected group.

3.1.2. Agrichemicals

Cortisol levels in fishes exposed to methyl-parathion and tebuconazole did not elevate in response to the injection of ACTH (ACTH group), with values significantly lower than the not exposed control fishes ($p<0.01$, Dunnett's test, figure 2, A and B). Fishes exposed to sub-lethal concentrations of Herbimix^{TM}, atrazine, and glyphosate showed a rise in plasma cortisol very similar to the control fish. Subsequent to the exposure of these three agrichemicals, the cortisol values found in the ACTH-injected fishes were higher than those measured in the saline-injected and non-injected groups ($p<0.01$, using Tukey's multiple range test).

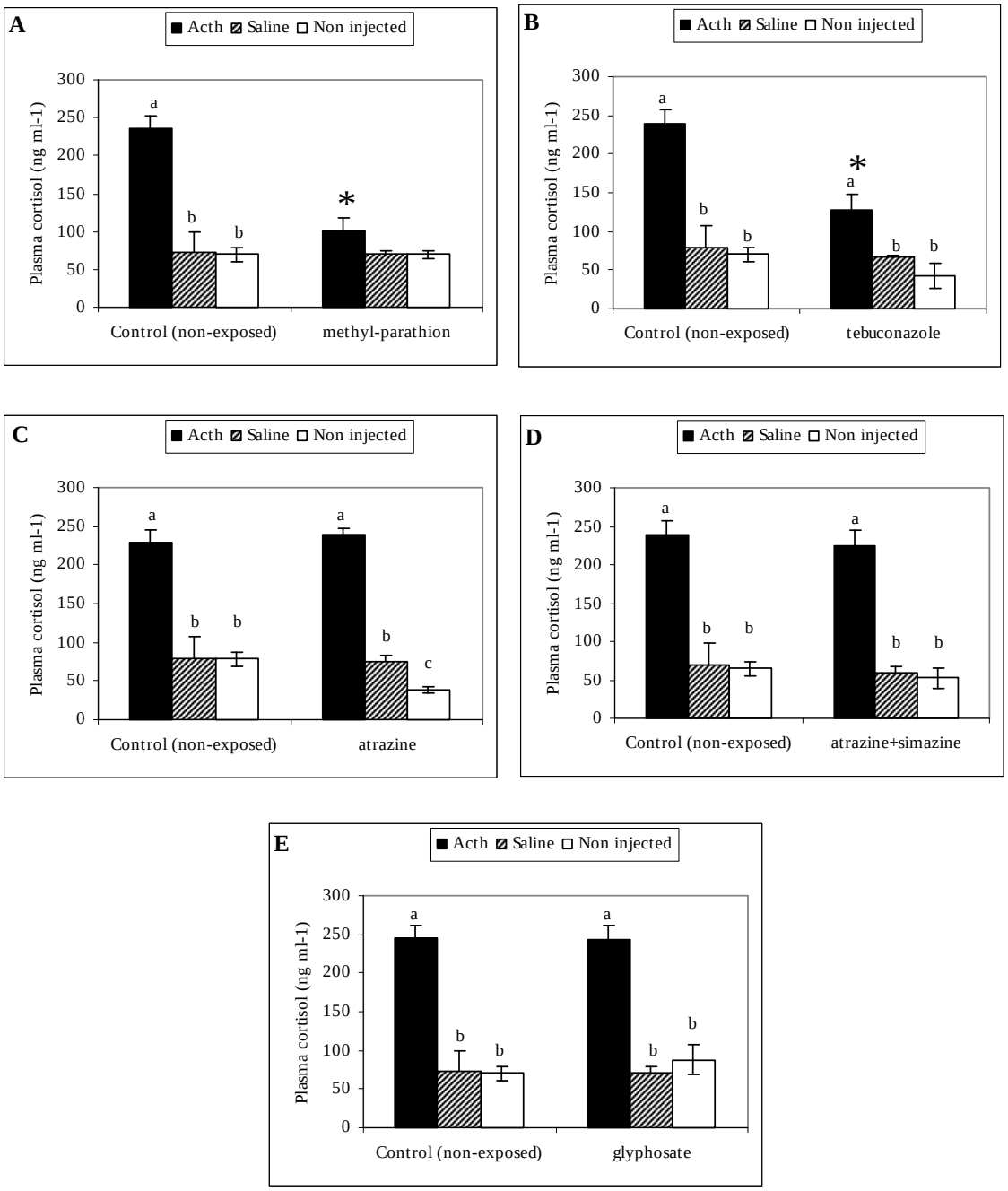

Figure 1. Plasma cortisol levels (mean ± SEM) in *Rhamdia quelen* subjected to ACTH injection (solid bars), vehicle injection (saline, traced bars), and in the non-injected fish (white bars) in control fish (non-exposed) and after acute exposure (96 h) to 16.6% of the $LC_{50\text{-}96h}$ of methyl-parathion (A), tebuconazole (B), atrazine (C), atrazine+simazine (D) and glyphosate (E). The asterisks indicate statistical difference to control value (ANOVA and Dunnett's tests, $p<0.05$). For each experiment (agrichemical) the different letters above the bars indicate statistical differences between different groups (ANOVA and Tukey's tests, $p<0.05$). (n=15).

4. DISCUSSION

Our earlier study investigated the impairment of the cortisol stress response in *R. quelen* exposed to five agrichemicals, using a standard acute stressor protocol (Cericato et al., 2008). In the present study, the functional integrity of the cortisol-secreting interrenal tissue was investigated in the same species using an in vivo ACTH challenge test.

The first observation is the typical rise in cortisol levels verified after a standardized i.p. injection of porcine $ACTH^{1-39}$ in all control fishes (i.e., in the fishes not exposed to agrichemicals). These values were very similar to results previously reported for the same species in response to acute stressors (Barcellos et al., 2001, 2003, 2004). As expected, the i.p. injection of the vehicle alone (the saline group) did not elicit a cortisol rise, with values very similar to the non–injected group.

The major result of this study is the finding of the ACTH challenge test, which revealed that the interrenal tissue of *R. quelen* exposed to 16.6% of the $LC_{50\text{-}96h}$ (Kreutz et al., 2008) of methyl-parathion and tebuconazole had a significantly reduced ability to respond to ACTH, compared with the fishes from the non-exposed group.

This interrenal dysfunction may be responsible for the impaired ability of *R. quelen* that are exposed to methyl-parathion to elevate plasma cortisol after acute stimulation caused by acute chasing stress (Cericato et al., 2008).

The ACTH challenge test artificially mimics the pituitary's function of releasing ACTH. This protocol allows us to affirm that the effect of methyl-parathion and

tebuconazole as endocrine disruptors within the HPI axis is located in the interrenal tissue. Though the experimental design of our work does not permit the precise determination of how these agrichemicals affect interrenal tissue, there are some possible explanations. Our first hypothesis, which is the focus of our continuing research, is that methyl-parathion and tebuconazole cause oxidative stress in interrenal cells, which is postulated as a possible cause of endocrine disruption by Dorval et al. (2005). As methyl-parathion has oxidative stress-inducing potential (Monteiro et al., 2006), which includes renal impact (Singh et al., 2006), the possible effects of oxidative stress in the mechanism of impairment of the cortisol response in *R. quelen* should be considered.

Another hypothesis is that the possible adrenotoxicity of methyl-parathion and tebuconazole may affect some steps of cortisol synthesis and release. Benguira et al. (2002) have demonstrated that rainbow trout subjected to a single injection of ortho-para-dichlorodiphenyldichloroethane (o-p'-DDD) lost their capacity to elevate plasma cortisol after confinement stress only seven days post injection. The authors attributed this dysfunction to the adrenotoxic effect of o-p'-DDD that might be mediated by alterations in the generation of cyclic adenosine monophosphate (cAMP) in cortisol synthesis and release by interrenal cells (Lacroix and Hontela, 2003). Hepatocytes transform methyl-parathion into methyl-paroxon, a more toxic metabolite (Loomis and Hayes, 1996), and since this metabolite is slowly inactivated in fishes as compared to mammals (Areechon and Plumb, 1990), the methyl-paroxon may exert an adrenotoxic effect, provoking the impairment of the cortisol response. This could explain the block in cortisol response to handling, four days after exposure to sub-lethal concentrations of methyl-parathion. To confirm, methyl-parathion causes hystopathological abnormalities as early as 1 h after contamination in *Corydoras paleatus* (Fanta et al., 2003). Abnormalities at the cellular

level were also verified in *Mystus cavasius* exposed to methyl-parathion (Murphy et al., 1984).

The third hypothesis is based on the evidence of HPI impairment after acute exposure to chemicals, as stated by Gravel and Vijayan (2007), who found inhibited adaptive plasma cortisol response and the associated metabolic changes in liver in trout that were given salicylate-laced feed for only three days. These authors postulated that the mode of action of salicylate involves disruption of steroidogenic acute regulatory (StAR) and liver glucocorticoid receptor (GR) proteins, which are critical for cortisol production and target tissue responsiveness to cortisol, respectively. A last hypothesis is that these agrichemicals altering intracellular receptors of ACTH impairing its signaling transduction.

Conversely, the impairment of the HPI axis, as verified by Cericato et al. (2008) in *R. quelen* exposed to atrazine + simazine and to glyphosate, was exerted in another point within HPI axis, as the interrenal tissue was fully responsive in the ACTH challenge test. In our previous work (Cericato et al., 2008), we hypothesized that the mechanism involved in the attenuation of the cortisol response to stress provoked by atrazine + simazine and glyphosate might be related to adrenotoxicity, but this study shows that the weakened cortisol response to acute stress, as verified in our previous study, may be related to a regulation of cortisol secretion in higher levels within the HPI axis (i.e., in the hypothalamus and/or in the pituitary gland).

Finally, atrazine did not show any effect that suggests endocrine disruption in the HPI, both in the stress test (Cericato et al., 2008) and the ACTH challenge test. Atrazine

can cause liver and kidney damage, and alters the liver enzymatic pathways in common carp (Neskovic et al., 1994) but, as the function of the organ was not seriously affected, this alteration could be considered a moderate pathological response. This could explain the fact that exposure to atrazine did not block the cortisol response to the ACTH challenge test. In contrast to our findings, adrenotoxic effects of atrazine were observed in amphibian species (Goulet and Hontela, 2003) and in rainbow trout (Bisson and Hontela, 2002).

In conclusion, the ACTH challenge test in our study revealed that *R. quelen* exposed to sub-lethal concentrations of tebuconazole and methyl-parathion had a reduced ability to elevate plasma cortisol in response to an i.p. injection of exogenous ACTH, indicating that the interrenal tissue is the site of the impairment within the HPI axis. These ACTH challenge tests also revealed that the impairment of the cortisol response verified in fishes exposed to atrazine + simazine and glyphosate (Cericato et al., 2008) seems to be related to the regulation of the cortisol secretion at higher levels within the HPI axis.

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5. CONCLUSÕES

5.1. Conclusão Geral

A exposição sub-letal a agrotóxicos exerce efeito sobre a resposta cortisolêmica em jundiá (*Rhamdia quelen*) e sensibilidade ao hormônio adrenocorticotrófico (ACTH).

5.2. Conclusões Específicas

- As concentrações sub-letais de atrazine na água não causa bloqueio no eixo HHI. No desafio pelo ACTH a resposta de elevação do cortisol, indica que o composto não causa qualquer tipo de interrupção endócrina no tecido interrenal.

- A concentração de 50% da CL50, tanto para os testes atrazine + simazine quanto para o glifosato, provocou um bloqueio no eixo HHI. No teste de desafio pelo ACTH, também para ambos os experimentos, o tecido interrenal respondeu de forma similar tanto em peixes expostos quanto em peixes controle, indicando que a inibição da elevação dos níveis de cortisol provocado pela dose 50% da $CL_{50\text{-}96h}$ parece não ser decorrente de efeito tóxico no tecido interrenal.

- A exposição aguda á concentrações sub-letais de metil-paration provoca inibição da resposta ao estresse e que esta interrupção endócrina ocorre no tecido interrenal, pois este não respondeu ao estimulo de ACTH.

- A exposição aguda a concentrações sub-letais de tebuconazole não provoca a inibição da resposta ao estresse em alevinos de jundiá. O tebuconazole provocou um efeito deletério no tecido interrenal mostrando uma diminuição da capacidade de síntese de cortisol em resposta a estimulação por ACTH.

- O teste de sensibilidade ao hormônio adrenocorticotrófico (ACTH) demonstrou que a alteração na resposta cortisolêmica ocorreu no tecido interrenal para os agrotóxicos atrazine + simazine, glifosato e tebuconazole e não para atrazine e metil-paration.