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Priscila Leocádia Rosa Dourado

Interferência do inseticida fipronil nas respostas ao estresse oxidativo de Tilápias do Nilo mediadas pelo ácido γ-aminobutírico (GABA), durante períodos de hipóxia.

São José do Rio Preto
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"É muito melhor lançar-se em busca de conquistas grandiosas, mesmo expondo-se ao fracasso, do que alinhar-se com os pobres de espírito, que nem gozam muito nem sofrem muito, porque vivem numa penumbra cinzenta, onde não conhecem nem vitória, nem derrota." (Theodore Roosevelt)".

RESUMO

O Regent®800WG é um dos inseticidas mais utilizados no cultivo de cana-de-açúcar no Brasil e apresenta como princípio ativo o fipronil. O fipronil apresenta alta capacidade de interação com os receptores do ácido gama-aminobutírico (GABA), sendo que seu principal mecanismo de ação é competir com o GABA pelos canais de cloro, resultando em excitabilidade. O fipronil é um composto persistente no solo e tem sido amplamente detectado em ambientes aquáticos. Em ambientes naturais a ação e efeitos desse tipo de composto podem ser influenciados por diferentes aspectos ambientais, como variações na temperatura da água, pH, salinidade e oxigênio dissolvido. Da mesma forma, os efeitos de poluentes ambientais podem alterar as adaptações dos animais frente a essas variações ambientais, elevando os riscos de mortalidade frente adversidades do ecossistema. Assim, o presente trabalho objetivou avaliar a influência da exposição a uma formulação comercial do fipronil (Regent®800WG) em peixes expostos à hipóxia e à diferentes temperaturas, utilizando parâmetros de estresse oxidativo, danos ao DNA, níveis de transcrição gênica, bem como concentração de hormônios sexuais. No primeiro estudo objetivou avaliar a influência da exposição ao fipronil (Regent®800WG) nas concentrações 0,1 e 0,5 $\mu g.L^{-1}$, no encéfalo, em parâmetros de estresse oxidativo nas brânquia e fígado e os efeitos genotóxicos em eritrócitos de *Oreochromis niloticus* submetidos à hipóxia for 3 e 8 horas. Em um segundo trabalho, objetivamos avaliar a influência do fipronil administrado na comida em respostas bioquímicas de robalos (*Dicentrarchus labrax*) sob diferentes temperaturas. Os resultados mostraram que os sistemas de defesa antioxidantes dos peixes permaneceram elevados durante as primeiras 3 horas de exposição, possivelmente para se adaptar às condições de estresse hipóxico. Nos grupos expostos ao fipronil, as concentrações e os tempos de exposição influenciaram o sistema de defesa antioxidante, alterando a atividade das enzimas antioxidantes CAT, GR e da enzima de biotransformação GST. Os resultados também nos permitiram observar que os mecanismos de desintoxicação foram específicos para cada tecido, sendo as alterações mais representativas encontradas nas brânquias. No cérebro, observamos que os níveis de transcritos dos receptores GABA, do HIF-1A e da enzima antioxidante CAT, mostraram um regulação negativa

para todos os grupos após 3 h de exposição, porém, no decorrer do tempo de exposição os níveis de transcrição mostraram voltar aos níveis basais, exceto no receptor GABA-b1 no grupo exposto ao fipronil (0,5 $\mu g.L^{-1}$) na presença de hipoxia. Os resultados do estudo feito com robalos (*D. labrax*) reveleram que o fipronil administrado na comida, sob a influência de diferentes temperaturas, prejudicou as respostas metabólicas e antioxidantes dos animais expostos, bem como influenciou na regulação dos hormônios sexuais (E_2, T, 11-KT). Esses resultados sugerem uma resposta do corpo do animal para balancear os efeitos negativos iniciais dos tratamentos, que possivelmente foram afetados pela presença de contaminantes ou das variáveis ambientais (hipóxia, temperatura). A exposição aos estressores ambientais combinados, causou maior distúrbio nas respostas bioquímicas, fisiológicas e neuronais dos peixes quando comparados aos fatores individuais, bem como foram capazes de induzir dano genotóxico e peroxidação lipídica nos tecidos dos peixes, reforçando a importância de avaliar os prejuízos que contaminantes químicos podem causar à espécies não-alvo, como peixes, e melhor compreender como esses contaminantes interferem nos mecanismos adaptativos dos peixes à variações nas condições ambientais.

Palavras-chave: pesticidas, efeitos ambientais, neurotoxicidade, biomarcadores, peixes.

ABSTRACT

Regent®800WG is one of the most used insecticides in the cultivation of sugarcane in Brazil and has as active principle fipronil. Fipronil exerts its insecticidal activity by competing with GABA for GABA-a receptor binding sites, blocking chloride channels and preventing the transmission of normal nervous impulse, which leads to inhibition of neuronal transmission and induces neuronal hyperexcitation. Fipronil is a persistent soil compound and has been widely detected in aquatic environments. In natural environments the action and effects of this type of compound can be influenced by different environmental aspects, such as variations in water temperature, pH, salinity and dissolved oxygen. Similar, the effects of environmental pollutants can modify the adaptations of the animals to these environmental variables, raising the mortality risks face to adversity in the ecosystem. Thus, this study aimed to investigate the influence of fipronil (Regent®800WG) in fish exposed to hypoxia and different temperatures, on the oxidative stress parameters, DNA damage, gene transcription levels, as well sex hormones levels. Our first study investigated the influence of fipronil (0.1 $\mu g.L^{-1}$ and 0.5 $\mu g.L^{-1}$) in the brain, in oxidative stress markers of gills and liver, and genotoxic effects on erythrocytes of Oreochromis niloticus submitted to hypoxia for 3 and 8 hours. In our second study, we aimed to evaluate on the effects of food administration of Fipronil (Regent®800WG) under two temperatures was carried out in the sea bass (Dicentrarchus labrax) for 21 days. Our results showed that the antioxidant defense systems in gills and liver of the fish remained elevated during the first 3 hours of exposure, probably to adapt to hypoxic stress conditions. In the groups exposed to fipronil, the concentrations and times influenced the antioxidant defense system, altering the antioxidant enzymes activity (CAT and GR), and the GST biotransformation enzyme. The results also allowing us to observe that the mechanisms of detoxification were specific for each tissue, and the most representative alterations were found in the gills. In the brain, the levels of GABA, HIF-1A, and antioxidant enzyme CAT transcript levels showed negative regulation for all groups after 3 h of exposure, however, after 8 h the transcript levels showed a return to control levels except for GABA-b1 receptor in

the group exposed to fipronil (0.5 μg.L^{-1}) in the presence of hypoxia. The results of the study with sea bass (D. labrax) revealed that fipronil administered in food under influence of different temperatures, impaired the metabolic and antioxidant responses of exposed animals, as well influenced the regulation of sex hormones (E_2, T, 11-KT). These results suggest a response by the animal body to balance the initial negative effects of treatments, which could be affected by the presence of contaminants or environmental variables (hypoxia, temperature). Our results showed that the exposure to environmental stressors combined caused greater disturbances in the biochemical, physiology and neuronal responses of the fish when compared to the individual factors, as well to induce genotoxic damage and lipid peroxidation in fish tissues, reinforcing the importance of evaluating the damage which chemical contaminants can cause to non-target species such as fish, and better understand how these contaminants interfere with the adaptive mechanisms of fish to stressful situations such as hypoxia.

Keywords**:** *pesticides, environmental effects, neurotoxicity, biomarkers, fish*

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INTRODUÇÃO

1. INTRODUÇÃO GERAL

1.1. *Poluentes ambientais, fipronil e fatores ambientais de estresse*

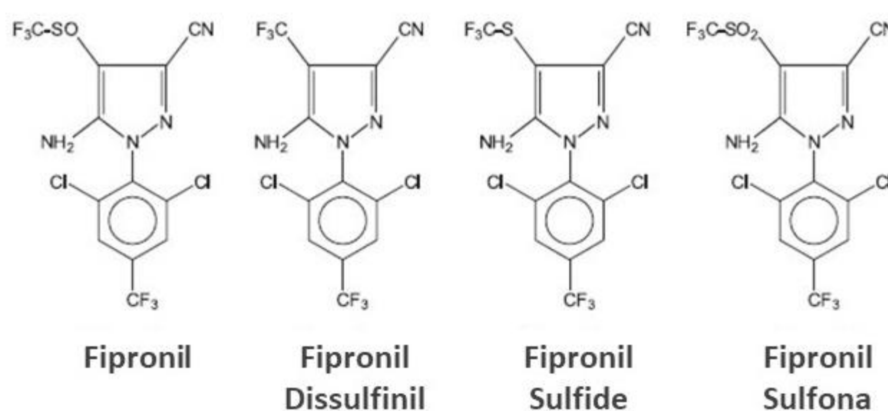
Diferentes tipos de compostos tais como metais, agrotóxicos, organoclorados, hidrocarbonetos e fármacos são produzidos e usados pelo homem, sendo posteriormente descartados no ambiente, possibilitando a contaminação dos recursos hídricos (POMA et al., 2012). Dentre essas diversas substâncias, os agrotóxicos se destacam pelo seu amplo uso em culturas agrícolas (ZACHARIA, 2011). O Brasil é o terceiro maior exportador de produtos agrícolas do mundo, sendo o principal produtor de açúcar, café e suco de laranja e o segundo maior produtor de soja, tabaco e etanol (ABRASCO – Associação de Saúde Coletiva). Segundo a ANVISA (2017), existem aproximadamente 600 principíos ativos diferentes de agrotóxicos utilizados na agricultura e o Brasil é considerado campeão mundial em consumo desde 2008, ultrapassando em 2010 um milhão de toneladas de agrotóxicos destinados ao cultivo soja, milho e cana-de-açúcar.

O Estado de São Paulo destaca-se como principal produtor de cana-de-açúcar, e dentre as usinas existentes no Brasil, 128 estão localizadas nesse Estado e com isso a atividade canavieira se torna responsável por pelo menos 20% do uso de agrotóxicos (SINDAG, 2006; ÚNICA, 2008). Em geral, um dos principais problemas associados aos agrotóxicos é à utilização irregular e/ou extensiva em locais próximos à ambientes aquáticos, que em razão do escoamento superficial causado pela água da chuva, afeta não só as espécies alvos desse produto químico nas lavouras, mas também as não-alvo dos ecossistemas aquáticos, como peixes (MARKAVERICH et al., 2002; VAN DER OOST et al., 2003). Estudos têm demonstrado que a presença de agrotóxicos favorece a contaminação do ambiente e pode afetar as taxas de crescimento e reprodução (ALMEIDA et al., 2018), mudanças bioquímicas e fisiológicas (MARKAVERICH et al. 2002; VAN DER OOST; BEYER; VERMEULEN, et al. 2003) e comportamentais nos organismos aquáticos (BOSCOLO et al., 2017).

Dentre os inseticidas utilizados na cana-de-açúcar no Estado de São Paulo, destaca-se o inseticida fipronil (5-amino-1-[2,6-dicloro-4(trifluormetil)fenil]-4-[(trifluorometil) sulfinil]-1H-pirazol-3-carbonitrila). Derivado quimicamente da família do fenil-pirazol, sua classificação toxicológica é (Classe II), é um dos

inseticidas mais utilizados no controle de insetos prejudiciais à cultura de cana-de-açúcar, inclusive os resistentes aos inseticidas piretróides, organofosforado e carbamato (STEFANI MARGARIDO et al., 2013). A degradação do fipronil pode ocorre por meio de uma reação de fotólise, hidrólise ou de oxigenação e redução, gerando produtos considerados altamente tóxicos e com alta atividade inseticida quando comparados ao composto original (LU et al., 2010), que são o fipronil sulfona e o fipronil dissulfinil (Fig. 1).

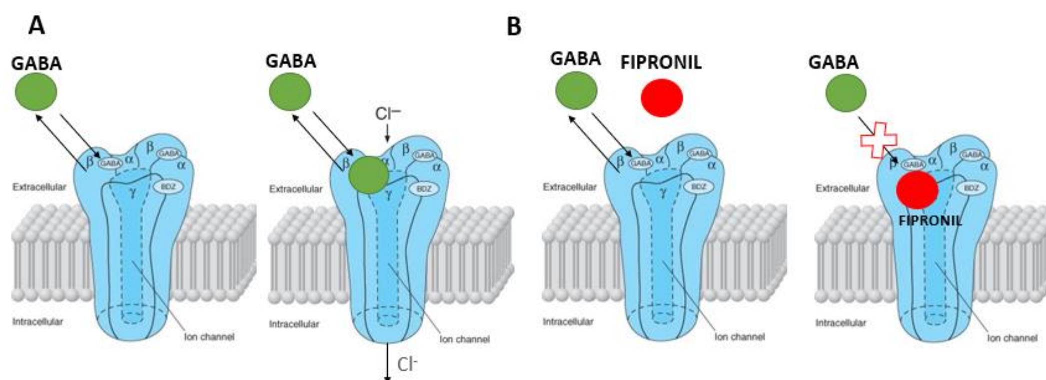
Figura 1 - Estrutura do fipronil e seus principais metabólitos (TKACH et al., 2017, com modificações).



Nos peixes, o fipronil, é bioconcentrado, ou seja, ocorre um aumento imediato no corpo do animal, assim que é absorvido do meio circulante, sendo que o grau de toxicidade varia de acordo com a espécie e a concentração exposta, podendo ser letal para a maioria dos peixes, principalmente os mais jovens (RAND; PETROCELLI, 1985). O fipronil apresenta alta capacidade de interação com os receptores ácido gama-aminobutírico (GABA), sendo que seu principal mecanismo de ação é competir com o GABA pelo canais de cloro, resultando em excitabilidade (BEGGEL et al., 2012) (Fig. 2). O GABA é um dos neurotransmissores mais importantes do sistema nervoso central, cerca de 20-50% das sinapses são GABAérgicas (RUDOLPH; MOHLER, 2004). A afinidade de ligação do fipronil aos receptores GABA é mais conhecida em insetos, mas alguns estudos indicam sua possível ligação aos receptores de vertebrados, resultando em efeitos neurotóxicos (KEGLEy et al., 2008; ZHANG et al., 2018). Muitos estudos têm demonstrado ainda efeitos significativos do fipronil na

produção de espécies reativas de oxigênio (ERO) e mudanças no mecanismo de biotransformação de animais aquáticos (STEFANI-MARGARIDO et al., 2013; WU et al., 2014; WANG et al., 2016; GRIPP et al., 2017).

Figura 2. (**A**) Interação do neurotransmissor GABA com o sítio de ligação do receptor GABA-a, permitindo a abertura dos canais iônicos e aumentando a permeabilidade dos íons cloreto, hiperpolarizando a célula. (**B**) Fipronil competindo com o GABA pelos sítios de ligação do receptor GABA-a, bloqueando os canais de cloreto e impedindo a transmissão do impulso nervoso normal, o que leva a inibição da transmissão neuronal. (Fonte: KATZUNG, 2017, com modificações baseadas no texto de KIDD; JAMES, 1991).



Em ambientes naturais a ação e efeitos desse tipo de composto podem ser influenciados por diferentes aspectos ambientais, como variações na temperatura da água, pH, salinidade e oxigênio dissolvido, que são variáveis críticas para a sobrevivência e adaptação das espécies (LYONS et al., 2011; HIEBENTHAL et al., 2013; FREITAS et al., 2017). Da mesma forma, os efeitos de poluentes ambientais podem alterar as adaptações dos animais frente a essas variações ambientais, elevando os riscos de mortalidade frente adversidades do ecossistema (NOYES et al., 2009).

Existem poucos estudos que avaliam a toxicidade do fipronil em peixes, no entanto, alguns trabalhos indicaram que o fipronil foi letal para as espécies *Oncorhynchus mykiss* (truta arco-íris) e *Lepomis Macrochirus* ("Bluegill") na concentração de 0,248 mg/L^{-1} e 0,130 mg/L^{-1}, respectivamente. Para a espécie *Oreochromis niloticus* (tilápias) o fipronil apresentou letalidade na concentração de 0,25 mg/L^{-1} (USEPA, 1996) e para a espécie *Poecilia reticulata* (guarú) na

concentração de 0,09 mg/L^{-1} (MANRIQUE et al., 2013). Alguns estudos demonstraram ainda a ação potencial do fipronil como desregulador endócrino em vários organismos, como peixes (ANKLEY et al., 2009; BEGGEL et al., 2012; BENCIC et al., 2013), crustáceos (VOLZ; CHANDLER, 2004) e mamíferos (LEGHAIT et al., 2010). O fipronil e outros inseticidas podem interferir no sistema endócrino dos animais e causar ruptura na produção e na ação dos hormônios naturais, pois se ligam ao receptor de estrogênio e alteram a função reprodutiva nos organismos expostos (MNIF et al., 2011). A exposição de larvas de peixes de água doce (*Pimephales promelas*) por um curto período ao inseticida Fipronil, causou aumento da regulação da produção de vitelogenina e retardou o desenvolvimento de células germinativas em larvas masculinas, provavelmente porque o fipronil afeta o sistema endócrino, em particular o estrogênico, alterando o desenvolvimento gonadal destes peixes (BEGGEL et al., 2012). Alterações no sistema endócrino de peixes por estrogênios ambientais, como o fipronil, têm causado grande preocupação, principalmente por seus mecanismos de ação na função reprodutiva do organismo, uma vez que mudanças na sinalização endócrina levam à falha reprodutiva (BENIC et al., 2013). Assim, a ação do Fipronil como disruptor endócrino pode ser aplicada como parâmetro para avaliar os sinais fisiológicos, como o crescimento e desenvolvimento do organismo (FILBY et al., 2006).

Apesar de haver algumas pesquisas que indiquem a toxicidade do fipronil em diferentes espécies (MNIF et al., 2011; TERÇARIOL; GODINHO, 2011; GRIPP et al., 2017), ainda existe carência de informações sobre os possíveis efeitos bioquímicos e fisiológicos dos peixes e de sua ação neurotóxica, especialmente considerando concentrações ambientalmente relevantes e combinação com outros fatores ambientais de estresse, como variações na temperatura da água, pH e oxigênio dissolvido.

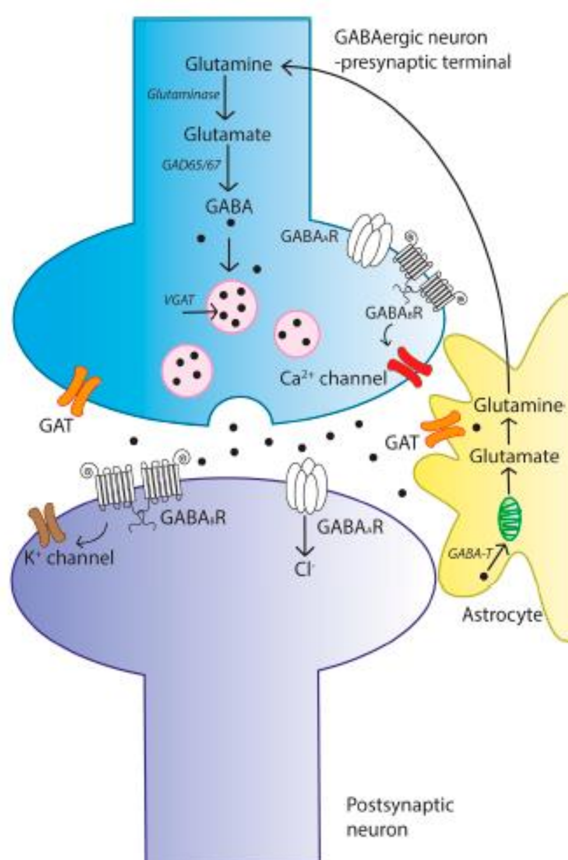
1.2. Hipóxia, estresse oxidativo e modulações GABAérgicas no encéfalo

Algumas espécies de peixes podem sobreviver a baixíssimas concentrações de oxigênio dissolvido, como é o caso da tilápia do Nilo (*Oreochromis niloticus*), que suportam situações de hipóxia (0,3 mg L^{-1} de oxigênio dissolvido) (DELANEY; KLESSIUS, 2004). Por outro lado, a maioria das espécies necessitam em torno de 5 mg L^{-1} de oxigênio dissolvido na água (FIORUCCI; FILHO, 2005). Mesmo

sobrevivendo às baixas concentrações de oxigênio dissolvido as tilápias desencadeiam adaptações bioquímicas e fisiológicas para proteger as células dessas condições, evitando a morte (ZENTENO-SAVÍN et al. 2002; LIGHTON; SCHILMAN, 2007). No entanto, a eficácia de tais mecanismos de adaptação à hipóxia é dependente do bom estado de saúde dos animais. Desta forma, podemos sugerir que alterações metabólicas nos animais, em decorrência da exposição a poluentes ambientais, podem afetar negativamente nas respostas adaptativas dos peixes à situações de hipóxia.

É sabido que em animais tolerantes à hipóxia, o GABA exerce papel fundamental na proteção do encéfalo contra os prejuízos que essa condição desfavorável pode ocasionar (GINNEKEN et al., 1996). Já foi evidenciado que peixes apresentam um aumento nos níveis de GABA encefálicos durante períodos de hipóxia, assim como aumento dos receptores GABA, de forma a reduzir o influxo de íons e diminuir o consumo energético do cérebro (LUTZ; NILSSON, 1997; NILSSON; RENSHAW, 2004). Alguns autores sugerem que o aumento dos níveis desse neurotransmissor ocorre como uma consequência do baixo aporte de oxigênio no sistema nervoso durante a hipóxia (NILSSON; LUTZ, 1993; KUMAR; 2011). Tendo em vista que a degradação do GABA é dependente de oxigênio, pode-se sugerir, então, que o neurotransmissor permanece por mais tempo no encéfalo, o que aumenta sua concentração ao longo do tempo (LUTZ et al., 1996). Assim, para potencializar o efeito da concentração do GABA nas fendas sinápticas, um aumento das concentrações dos receptores do GABA deve ocorrer, levando a uma diminuição geral do metabolismo neuronal (LUTZ; NILSSON, 1996). Assim, a explicação para o aumento dos níveis de GABA e seus receptores nesses animais tolerantes à hipóxia (esse efeito não é observado em mamíferos, por exemplo), teria como explicação, uma forma de proteger o cérebro, fazendo com que seu metabolismo reduza, assim evitando maiores gastos energéticos. Em consonância com essa hipótese, Nilsson & Renshaw (2004) mostraram que esse estímulo da produção do GABA é ainda maior caso os animais estejam privados de energia, o que também demonstra que essa resposta não é apenas uma consequência da falta de oxigênio para as enzimas envolvidas na degradação do GABA.

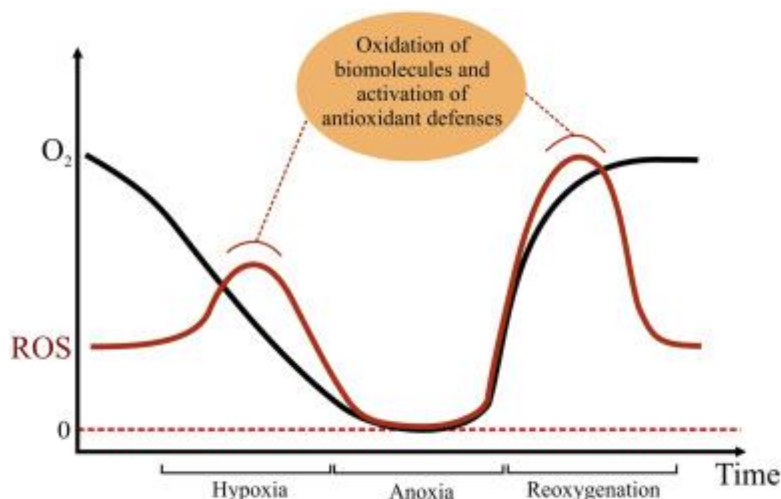
Figura 3 - Visão simplificada do sistema de sinalização do ácido γ-aminobutírico (GABA) e descreve os principais aspectos de uma sinapse gabaérgica. O GABA é sintetizado no terminal pré-sináptico a partir do glutamato, pela ação da decarboxilase do ácido glutâmico (GAD). O GABA é então recrutado para as vesículas sinápticas através da ação do transportador GABA vesicular (vGAT). Após a despolarização da membrana, a liberação do GABA ocorre por exocitose e depende da liberação do Ca^{+2} e pode ligar-se tanto aos receptores ianotrópicos (GABA-a, c) como os metabotrópicos (GABA-b), resultando em inibição do neurônio pós-sináptico. Após a sinapse, o GABA liberado é eliminado pela ação de transportadores de GABA ligados a membrana (GATs) localizados nos neurônios e astrócitos. Nos astrócitos, o GABA é reciclado em vesículas sinápticas ou absorvido pelas mitocôndrias, onde é convertido em metabólitos do ciclo de Krebs e novamente ser convertido em glutamato. (Fonte: imagem e texto baseados em revisão publicada por GOVINDPANI et al., 2017).



Estudos mais recentes indicam que tanto a produção do GABA quanto a expressão dos receptores do GABA podem ser estimulados pelo aumento na produção de espécies reativas de oxigênio (ERO) na mitocôndria de animais e cloroplastos de

vegetais (MARUTA et al, 2013; GONZÁLES et al., 2014). Ainda que não existam relatos que confirmem esse mecanismo em situações de hipóxia, é provável que o aumento da produção do GABA e seus receptores no encéfalo nessas situações seja mediado por aumento na produção de ERO durante a hipóxia. A produção de ERO durante períodos de hipóxia é um quadro já bastante conhecido na literatura (Fig. 4) (HERMES-LIMA; ZENTENO-SAVIN, 2002; ALMEIDA et al. 2005; GOROKHOVAA et al. 2012; WELKER et al. 2013; NOGUEIRA et al., 2017). As ERO podem servir como moléculas sinalizadoras que ativam mecanismos de respostas à hipóxia, incluindo a ativação do fator de indução de hipóxia (HIF), que culmina na ativação de respostas metabólicas de proteção contra os efeitos negativos da hipóxia, incluindo a expressão de genes de enzimas de defesa antioxidantes (CHANDEL et al., 2000). O aumento das defesas antioxidantes geradas durante a hipóxia está relacionado tanto à proteção inicial contra a formação de ERO, como poderia ocorrer para deixar o organismo previamente preparado para o aumento excessico de ERO, durante os períodos posteriores de reperfusão dos tecidos, quando as concentrações adequadas de oxigênio são restabelecidas (HERMES-LIMA et al., 2015), assim evitando o estresse oxidativo. Estudos mostraram que em condições hipóxicas há o aumento dos potenciais antioxidantes em animais aquáticos (Welker et al., 2013). Lushchak & Bagnyukova (2006), por exemplo, mostraram que a carpa comum (*Cyprinus carpio*) sob hipóxia por 5 horas apresenta aumento nas atividades das enzimas Catalase e Glutationa Peroxidase no cérebro.

Figura 4 - Modulação das defesas antioxidantes para se preparar para o estresse oxidativo. De acordo com essa teoria, em algum momento durante a hipoxia, ocorre um aumento temporário da formação de espécies reativas de oxigênio (ERO) mitocondriais. Além disso, durante a reoxigenação, conforme a concentração de oxigênio aumenta, também há formação de ERO. Em ambos os momentos, é esperado que essas moléculas causem danos oxidativos e aumente as defesas antioxidantes, ou seja, agem como moléculas sinalizadoras para preparação ao estresse oxidativo. (Fonte: imagem e texto baseados de revisão publicada por HERMES-LIMA et al., 2015).



Os efeitos da produção de ERO na sensibilidade da neurotransmissão gabaérgica são de grande importância, pois as ERO geradas podem interagir com os receptores do GABA direta ou indiretamente (via peroxidação lipídica, por exemplo) e com proteínas de transporte de íons, alterando a atividade do receptor e o balanço iônico, importante na manutenção dos potenciais de membana dos neurônios (SAH et al., 2002). Há relatos de que o aumento das concentrações de GABA assim como de seus respectivos receptores, modulam positivamente a atividade de enzimas antioxidantes, causando proteção do cérebro contra o estresse oxidativo (KURUVILLA et al., 2013; DIAS et al., 2014; XIE et al., 2015). O GABA, portanto, poderia ser um importante modulador das defesas antioxidantes celulares em situações de hipóxia, e qualquer desregulação na produção desse neurotransmissor e/ou atuação nos seus respectivos receptores encefálicos, poderia afetar as respostas antioxidantes encefálicas do organismo, tornando-o mais suscetível ao estresse oxidativo.

Nesse contexto, tendo em vista que a presença de compostos químicos no ambiente, como o fiponil, pode alterar o sistema de defesa antioxidante e causar danos às macromoléculas induzindo o estresse oxidativo (CLASEN et al., 2012, WEIDINGER; KOZLOV, 2015), conforme demonstra em vários estudos em peixes (BEGGEL et al., 2012; WU et al., 2014; WANG et al., 2016), é possível que os mecanismos de adaptação das espécies a essas condições de hipóxia sejam afetados. No entanto, estudos focando a influência de poluentes em respostas adaptativas à hipóxia são ainda inexistentes na literatura científica.

1.3. Influência da temperatura em respostas fisiológicas a poluentes

As atividades humanas tem sido considerada um grande problema para as comunidades aquáticas, uma vez que os compostos utilizados ou gerados à partir dessas atividades, têm demonstrado serem capazes de influenciar e alterar os parâmentros físicos, como pH e temperatura (HOOPER et al., 2013). Os efeitos causados por variáveis ambientais, em especial mudanças na temperatura, tem sido caracterizado por consequências para a vida silvestre e para o homem (HOOPER et al., 2013). Diversos estudos demonstraram que o aumento da temperatura causa impacto na acidificação dos oceanos induzida pelo CO_2, o que pode modificar a tolerância térmica dos ectotérmicos marinhos (PÖRTNER; FARRELL, 2008; LANNIG et al., 2010). Além disso, já foi relatado que exposição a mudanças na temperatura natural da água é capaz de alterar parâmetros na atividade de enzimas hepáticas (EROD) em *Oreochromis niloticus* (AMUTHA; SUBRAMANIAN, 2010). Essas alterações na temperatura causam mudanças na atividade de enzimas antioxidantes (CAT, GPx, GST) e alterar os níveis de peroxidação lipídica (MDA) em diferentes espécies de peixes (VINAGRE et al., 2012; MADEIRA, et al., 2013; MACHADO et al., 2014). No entanto, os efeitos indiretos causados por mudanças na temperatura em espécies aquáticas, em relação ao potencial de interação dessas alterações com produtos químicos, por exemplo, ainda são pouco conhecidos (HOOPEr et al., 2013). A mudança de fatores físicos em conjunto com exposições químicas pode agir sinergicamente e aumentar as consequências das exposições a organismos aquáticos, incluindo peixes e bivalves (SOKOLOVA; LANNIG, 2008; CARREGOSA et al., 2014; FREITAS et al., 2015). Assim, há um consenso atual da comunidade científica para se avaliar as possíveis consequências de alterações dos fatores abióticos, principalmente frente à exposição química em ambientes naturais, de forma a solucionar e identificar os efeitos para os seres humanos e a vida selvagem (WITTMANN; PÖRTNER, 2013).

Já tem sido discutido que o aumento da temperatura, causado pelas mudanças climáticas, influenciam no grau de toxicidade de muitos compostos químicos no ambiente, pois a interação da temperatura com a estrutura química do composto, ou vice-versa, pode afetar e modificar os processos de deposição e degradação nos compartimentos aquáticos (KIMBERLY; SALICE, 2013). A combinação do estresse

químico, proveniente de agrotóxicos da agricultura, com o estresse ambiental, devido aumentos na temperatura, por exemplo, podem resultar em efeitos mais acentuados ou aditivos aos organismos (HOLMSTRUP et al., 2010). Isso ocorre porque por um lado a exposição a contaminantes químicos pode reduzir a tolerância térmica dos organismos aquáticos, aumentando o risco de estresse por mudanças na temperatura. Além disso, mudanças na temperatura podem tornar os organismos mais susceptíveis à ação de contaminantes, já que também tem sido documentado que a temperatura influência na toxicidade de compostos químicos (PATRA et al., 2015). Por esse motivo, os organismos aquáticos tem sido apontados como os principais alvos do aumento da temperatura, principalmente em locais próximos à áreas agrícolas. Neste contexto, os animais desenvolvem diferentes mecanismos de compensações fisiológicas, comportamentais e morfológicas para lidar com mudanças ambientais naturais, e a presença de substâncias tóxicas no meio aquático podem interferir nesses ajustes normais dos animais às constantes do ambiente (MIDDLEBROOKS et al., 1973).

Para peixes de áreas estuarinas e costeiras, por exemplo, a temperatura da água é um fator abiótico básico que regula sua fisiologia e metabolismo, pois estão constantemente expostos a severas mudanças de temperatura e também salinidade e, por habitar áreas costeiras, acabam expostas à muitas substâncias derivadas de águas residuais (COSTAS et al., 2012; GAW et al., 2014; BLAIR et al., 2015; GONZÁLEZ-MIRA et al., 2016). Assim, a ação de compostos que chegam aos ambientes aquáticos pode ser influenciada por diferentes aspectos físicos, como temperatura (LYONS et al., 2011; HIEBENTHAL et al., 2013; FREITAS et al., 2015). Além disso, mudanças nos mecanismos de biotransformação de xenobióticos podem promover impactos significativos no controle de muitas funções corporais, tornando os animais mais vulneráveis. Tem sido relatado que várias isoenzimas da família P450 estão envolvidas no metabolismo da biotransformação de xenobióticos (WANG et al., 2016). A exposição a compostos químicos que induzem a biotransformação em conjunto com fatores ambientais é uma preocupação para os peixes, já que seu metabolismo pode ser regulado pela temperatura, e que muitas vezes não compensados pela ação enzimática (GONZÁLEZ -MIRA et al., 2016). Estudos também mostraram essa forte modulação da desintoxicação metabólica e xenobiótica em peixes *S. senegalensis* apenas por meio

da temperatura (SOLÉ et al., 2015); ou em combinação com exposições químicas (GONZALEZ-MIRA et al., 2016). Além disso, mudanças na temperatura da água também podem gerar mudanças nos níveis celular e molecular que podem ser avaliados e medidos por respostas bioquímicas.Assim, nesse cenário, acredita-se que as espécies aquáticas que habitam ou são cultivadas em baías próximas á áreas agrícolas estão cada vez mais ameaçadas pelos efeitos da exposição a poluentes locais e por modificações nos parâmetros físicos da água causados pelas mudanças climáticas.

1.4. Hipóteses do estudo do presente trabalho.

Considerando que o fipronil atua ligando-se competitivamente aos receptores do GABA, e que esse sistema possui grande relevância na proteção do organismo contra os efeitos negativos da hipóxia. A hipótese do presente estudo é que o fipronil por competir com GABA, bloqueará seus receptores, gerando uma resposta compensatória do sistema nervoso do animal. Essa resposta aumentará a transcrição de genes dos receptores e a síntese do GABA no encéfalo, de forma a tentar superar a falta de resposta desse neurotransmissor devido ao bloqueio dos receptores. A hipóxia, por sua vez, como forma de diminuir o metabolismo no encéfalo e os custos energéticos, causará aumento do GABA encefálico, para evitar prejuízos às células devido a falta de energia em hipóxia. Assim, tanto o fipronil quanto a hipóxia causarão aumento na geração de ERO, desencadeando possíveis danos ao DNA e peroxidação lipídica, bem como alterações na atividade (catalase, glutationa peroxidase, glutationa redutase, superóxido dismutase e glutationa S-transferase) e expressão gênica (SOD; CAT) de enzimas antioxidantes. Além disso, quando em conjunto, os efeitos do fipronil e da hipóxia aumentam substancialmente, interferindo na resposta dos peixes frente ao estresse, gerando mais efeitos deletérios. Ainda, considerando que alterações na temperatura da água ocasionam mudanças significativas na fisiologia dos peixes, nesse trabalho também testamos a hipótese de que aumentos na temperatura da água causarão alterações em marcadores fisiológicos relacionados ao metabolismo energético, estresse oxidativo, e enzimas de biotransformação, bem como na atividade de esterases de robalos (*D. labrax*), e que a exposição ao inseticida fipronil irá incrementar os efeitos deletérios da temperatura nesses marcadores.

OBJETIVOS

2. OBJETIVOS

2.1 Objetivo geral 1– Efeitos do fipronil e da hipóxia em tilápias (etapa desenvolvida no Brasil)

Avaliar a influencia da exposição ao fipronil (Regent®800WG) no encéfalo e em parâmetros de estresse oxidativo em brânquia e fígado de *Oreochromis niloticus* submetidos à hipóxia.

Objetivos Específicos:

- Avaliar a influência da exposição ao inseticida fipronil nas respostas de parâmetros de estresse oxidativo à hipóxia (enzimas antioxidantes e peroxidação lipídica), em fígado e brânquias de tilápias.
- Avaliar os efeitos genotóxicos isolados e combinados da hipóxia e da exposição ao fipronil em eritrócitos de *O. niloticus*.
-Avaliar a influência da exposição ao inseticida fipronil nas respostas encefálicas do GABA e de transcritos de receptores do GABA, do fator de indução de hipóxia (HIF) e das enzimas antioxidantes Catalase e Superóxido Dismutase, frente à hipóxia.

2.2 Objetivo geral 2 – Efeitos do fipronil e da temperatura em robalos (etapa desenvolvida em Barcelona)

Avaliar a influência do fipronil em respostas bioquímicas de robalos (*Dicentrarchus labrax*) sob diferentes temperaturas.

Objetivos específicos:

- Avaliar os efeitos do fipronil na fisiologia de *D. labrax* mantidos sob diferentes temperaturas por meio da análise da atividade de esterases (AChE, PrChE e BuChE) no músculo, atividade hepática da Lactato Desidrogenase (LDH), atividade da enzima de conjugação Glutationa S-Transferase (GST), bem como os níveis de peroxidação lipídica no fígado, atividade das enzimas antioxidantes (CAT, GPx e GR), e concentrações dos hormônios esteróides Estradiol (E_2), Testosterona (T) e 11-Cetotestosterona (11-KT) no plasma.

METODOLOGIA, RESULTADOS E DISCUSSÃO

3. METODOLOGIA, RESULTADOS E DISCUSSÃO

As metodologias e resultados serão apresentados em forma de capítulos individualmente, conforme as etapas realizadas nesse projeto de doutorado e levando em consideração os diferentes objetivos propostos. Os manuscritos estão de acordo com as normas das revistas selecionadas para submissão, seguindo as normas propostas pelo Programa de Pós-graduação em Biociências, do Instituto de Ciências Biociências, Letras e Ciências Exatas, UNESP/IBILCE.

Capítulo 1 – "**Fipronil alters GABAergic responses in the brain of Nile Tilapia during hypóxia".**

Capítulo gerado referente ao projeto de pesquisa "Interferência do inseticida fipronil nas respostas ao estresse oxidativo de tilápia -do- nilo mediadas pelo ácido γ-aminobutírico (GABA) durante períodos de hipóxia" (FAPESP 2015/15191-1). Os resultados apresentados nesse capítulo fazem referência aos efeitos da hipóxia e fipronil nos níveis do GABA e de transcritos (mRNA) de seus receptores no cérebro de tilápia (*Oreochromis niloticus*) expostas à hipóxia. Para esse capítulo, foram computados os resultados dos receptores GABA-a (α, β e γ), GABA-b (1 e 2) e GABA-c (Rho), além dos níveis de transcritos das enzimas antioxidantes CAT e SOD e os dados de quantificação do neurotransmissor GABA. Esses resultados foram submetidos para publicação no periódico *Journal of Environmental Management*.

Capítulo 2 – "**Genotoxic and oxidative stress markers in *Oreochromis niloticus* after isolated and combined exposure to hypoxia and fipronil."**

Capítulo gerado referente ao projeto de pesquisa "Interferência do inseticida fipronil nas respostas ao estresse oxidativo de tilápia- do-Nilo mediadas pelo ácido γ-aminobutírico (GABA) durante períodos de hipóxia" (FAPESP 2015/15191-1). Nesse capítulo abordamos os resultados referentes ao efeito da hipóxia e fipronil no sistema de defesa antioxidante nas brânquias e fígado de tilápia-do-Nilo. Os resultados obtidos nos proporcionaram observar que esses dois fatores tornam os peixes susceptíveis ao estresse oxidativo, devido a alterações na atividade de enzimas antioxidantes, nos níveis de MDA e danos ao DNA.

Capítulo 3 – **“Combined effects of the insecticide Fipronil (REGENT® 800WG) and temperature on the metabolism of the benthic fish *Dicentrarchus labrax*”.**

Capítulo gerado referente ao projeto de pesquisa “Combined effects of the insecticide Fipronil (REGENT® 800WG) and climate change factors on the metabolism of the benthic fish *Solea senegalensis*”, desenvolvido durante período de “Doutorado Sanduíche” (FAPESP 2017/18210-2) em parceria com o Instituto de Ciencias do Mar – Barcelona, sob supervisão da Dra. Montserrat Solé. Os resultados apresentados nesse capítulo ainda não estão apresentados no formato final para publicação, uma vez que ainda serão implementados outros resultados que ainda estão sendo finalizados pela equipe do Instituto de Ciências do Mar.

CAPÍTULO I

Effects of Fipronil on the GABAergic responses in the brain of Nile Tilapia under normoxia and hypoxia conditions.

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ABSTRACT

γ-aminobutyric acid (GABA) is one of the main neurotransmitter involved in the adaptation processes against the damages that hypoxia can cause to the brain in fish. The insecticide fipronil, due its antagonist action on GABA receptors, can turn the fish more susceptible to the negative effects of hypoxia. Thus, this study evaluated the effects of fipronil exposure on the GABAergic responses in the brain of Nile tilapia (*Oreochromis niloticus*) under normoxia and hypoxia conditions. *O. niloticus* were exposed for 3 and 8 hours to fipronil (0.0, 0.1 and 0.5 $\mu g.L^{-1}$) under normoxia (dissolved O_2 > 6 $mg.L^{-1}$) and hypoxia (dissolved O_2 < 2 $mg.L^{-1}$) conditions. In general and only at 3h exposure, the transcript levels of GABA receptors, HIF and CAT showed a marked down-regulation in groups exposed to fipronil when compared to the not exposed one, both under normoxia and hypoxia. We also observed decreased GABA levels in the fish submitted only to hypoxia, strongly suggesting a decrease in GABAergic neurotransmission in tilapias under 3h of hypoxia. Furthermore our study demonstrated a dbecrease in the GABA neurotransmitter and a downregulation of the β subunit of GABA-a receptor in the initial periods of hypoxia exposure, both parameters were back increase levels after 8h of hypoxia, except GABA-b1 receptor in the group exposed to hypoxia and fipronil at 0.5 $\mu g\ L^{-1}$, suggesting an adaptation response to the initial negative effects of treatments.

Keywords: Fipronil**,** GABA receptors, HIF-1A, Antioxidant enzymes, fish

1. Introduction

Environmental hypoxia, is a common phenomena in aquatic environments often caused by the excessive decomposition of organic matter in association with high temperatures (Zhang et al., 2013; Tiedke et al., 2014). Organisms that are frequently subject to oxygen deprivation have developed behavioral, physiological, biochemical and molecular adaptation mechanisms which permit them to survive under these adverse conditions (Roesner et al., 2006; Hermes-Lima et al., 2015). However, the presence of anthropogenic chemical contaminants in the aquatic environment can interfere within these adaptations, which might compromise organism homeostase. Nile Tilapia, have great ability to survive under low concentrations of dissolved oxygen (~2,0 $mg.L^{-1}$) due to several metabolic adjustments that leads cells to a decrease mitochondrial respiration (Delaney & Klessius 2004). However, the efficacy of these adaptative mechanisms depends on the health status of the animal (Lacetera, 2018), therefore any metabolic alteration in fish due to exposure to environmental pollutants may negatively affect their responses to hypoxia.

During hypoxia, as oxygen is a major determinant of cell metabolism, it is expected that the gene expression profiles and neurotransmitters levels in the cells are significantly altered, in a process initiated mostly by the hypoxia-inducible factor-1 (HIF-1) transcription factor (Solaini et al., 2010; Lunde et al., 2011). HIF-1 mediates adaptation of the fish to hypoxia by inducing the expression of glycolytic genes and thus altering the glycolysis oxidative metabolism, that prevent the conversion of pyruvate to acetyl-CoA, in a mechanism regulated by the enzymes lactate dehydrogenase A (LDH-A) and pyruvate dehydrogenase kinase 1 (PDK1) (Wheaton & Chandel, 2011). Aiming to repress mitochondrial function and oxygen consumption, HIF-1 induces PDK1 that phosphorylates and inhibits pyruvate dehydrogenase, increasing the conversion of pyruvate to lactate and reducing electron flux through the electron transport chain (ETC) (Gray et al., 2014). As a consequence, a drop in oxygen consumption by the mitochondria occurs (Papandreou et al., 2006).

It has been shown that the decrease in cellular ATP concentration due to low oxygen supply during hypoxia in hypoxia tolerant animals stimulates the increase of

γ-aminobutyric acid (GABA) in neurons, due to activation of glutamate decarboxylase (GAD), responsible for the anaerobic conversion of glutamate to GABA (Mangia et al., 2012). Moreover, the low ATP availability can also contribute to inhibit GABA transaminase (GAT), the main enzyme involved in the GABA degradation (Madl & Royer, 2000; Govindpani et al., 2017). Indeed, GABA degradation is dependent NAD+ and α-ketoglutarate, scarce substances in anaerobic situations (Lutz et al., 1996), thus further contributing to maintain increased GABA encephalic levels during hypoxic periods (Anju et al.,2010). As a consequence, an increase in the amount of GABA receptors can also occur in order to potentiate the effect of increasing the GABA concentration in synaptic clefts, leading to a general decrease in neuronal metabolism (Lutz & Nilsson, 1997).

The GABA-a receptors are the main binding site for neuronal inhibition by GABA, and mediates an increase in membrane conductance with an equilibrium potential, generally near to the resting level. Frequently is followed by a membrane hyperpolarization, which increases the potential for firing in the synaptic clefts and reduce in the probability of action potential to initiate, causing neuronal inhibition (Olsen & DeLorey, 1999). This reduction in neuronal metabolism may serve as protection for neurons against hypoxia, and is facilitated by the dependence of GABA for the inflow of Chlorine through a channel associated to the receptor (Olsen e Delorey, 1999). Therefore, increases of GABA levels and their receptors in these hypoxia tolerant animals (effects that are not not observe in mammals), could occur as a form of protection to the brain, reducing its metabolism and energy consumption.

Studies have indicated that both GABA production and GABA receptor expression can be stimulated by increased production of reactive oxygen species (ROS) in animal cells, as well as in plant chloroplasts (Maruta et al, 2013, Gonzáles, et al., 2014). Although there are no reports confirming this mechanism in hypoxia situations, it is likely that the increased production of GABA and its receptors in the brain would be mediated by increases in ROS production during hypoxia.

During hypoxia, despite the low molecular oxygen concentrations, ROS production can increase in mitochondria, specifically at the complex III of the electron transport chain (Chandel et al., 2000). It is believed that the formation of

hydrogen peroxide during this hypoxic state can act as cascade signaling molecule culminating in the induction of hypoxia inducible factor (HIF-1) (Boutilier, 2001). HIF is a heterodimer that consists of two stable subunits (HIF1-α and β), which are strictly regulated by the intracellular oxygen concentration (Park et al., 2010). In normoxia, these subunities are rapidly degraded in a hydrolysis reaction by the action of O_2 and Fe_2 (Salcedo & Caro, 1997). However, under hypoxic conditions, the enzymes responsible for transporting these elements are inactivated, resulting in stabilization and accumulation of HIF in the cytoplasm, that, subsequently, migrates to the nucleus and forms the HIF1 transcriptionally active complex, which then binds to elements of hypoxia within the promoter region of the gene and activates its expression (Wang et al., 1995; Ke & Costa 2006; Niebler et al., 2015). HIF is the main mediator of the adaptive responses of cells to changes in oxygen supply, and its role in several physiological and pathological processes has also been documented (Majmundar et al., 2010). Evidences has indicated that in situations of moderate hypoxia (1,5 % O_2) the mitochondria stimulate the production of reactive oxygen species (ROS), specifically in the electron transport complex III, which inhibits the activity of enzymes with prolyl hydroxylase domains (PHD) and promotes the degradation of HIF1-α, impairing its hypoxic stability (Klimova e Chandel, 2008; Majmundar et al., 2010).

The increase in the antioxidant defenses during hypoxia relates both to protection against ROS generated and could occur as a way of leaving the body previously prepared for ROS excessive production during subsequent periods of tissue reperfusion when adequate oxygen concentrations are reestablished (Hermes-Lima et al., 2015). It has been reported that increasing in the GABA concentrations, as well as their respective receptors, positively modulate the activity of antioxidant enzymes, causing brain protection against oxidative stress (Kuruvilla et al., 2013; Dias et al., 2014; al., 2015). GABA, therefore, seems to be an important modulator of cellular antioxidant defenses in hypoxia situations and any desregulation in its production and/or performance in its respective brain receptors could affect the antioxidant responses of the organism, making it more susceptible to oxidative stress.

However, it is known that the exposure of organisms to environmental variables, such as hypoxia, does not always occurred isolate, thus, the production of ROS can

also occur due to the exposure of organisms to chemical compounds in the environment, which causes changes in the antioxidant defense system, leading to damage in cellular macromolecules such as lipids, DNA and proteins, inducing oxidative stress (Clasen et al., 2012, Weidinger and Kozlov, 2015) and impairing the adaptation of the animals to the conditions of hypoxia. Thus, the intense use of agrochemicals in agriculture has been characterized as a potential problem for aquatic organisms (Abhijith e Gokhale, 2015). The contamination of water resources, due to the drain of these substances by rainwater, favors the contamination of the environment making the contaminants bioavailable to the fish and interfering in the normal survival and adaptation of the animals to the environmental constants (Markaverich et al. 2002; Van Der Oost et al. 2003; Ignácio 2014). In addition, because they are persistent in the environment and presented high potential for bioaccumulation, many agrochemicals are highly toxic that for fish even at concentrations below those recommended by regulatory agencies (Rand & Petrocelli 1985; Rahman et al., 2002). Thus, it is evident that exposure to chemical contaminants can take to biological consequences to fish and impair their normal adaptation to environmental variables.

The phenylpyrazole fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4(trifluoromethylsulfonyl)pyrazole-3-carbonitrileis) classified as highly toxic (Class II), is one of the most insecticides used in the control of insects harmful to sugarcane cultivation, including those resistant to pyrethroid, organophosphate and carbamate insecticides (Stefani Margarido et al. 2013). Its main action mechanism is to inhibit the normal nerve impulse of cells, for acting directly on the γ-aminobutyric acid (GABA) chloride channels in insects, disrupting neuronal signaling (Kidd & James 1991; Bloomquist 2005). Due to their higher affinity to GABA receptors for insects than mammals, has been widely used in veterinary medicine, given greater safety to vertebrates (Kegley et al. 2008). However, GABA antagonists such as fipronil are known to cause damages in non-target species, as birds (US EPA, 2012) and fish (Hayasaka et al., 2012; Bencic et al., 2013; Wu et al., 2014). In fish, the presence of fipronil has already been associated with changes in the activity of cytochrome P450 superfamily enzymes (Wu et al., 2014); negative effects on reproductive endocrinology (Bencic et al., 2013); oxidative stress (Classen et al., 2011; Menezes

et al., 2016; Wang et al., 2016) and changes on gene expression (CYP 3A126 and Vitellogenin) (Beggel et al., 2012). Therefore, the intensive use of fipronil in agriculture and veterinary medicine presents a risk to aquatic organisms, especially fish, living in risk areas, close to the places where the insecticide is used.

Given the role that GABA discharge in protecting the brain from hypoxia, the presence of chemical contaminants such as fipronil, which connects to chlorine channels effectively, blocking the passage of ions, can make the organisms more susceptible to the negative effects of hypoxia. Besides that, can affect negatively the cerebral protection of the animal in these conditions, modifying the action of antioxidant enzymes and interfering in the normal function of GABA in the brain. Moreover, little is known whether this mechanism of GABA brain protection in fish also helps to protect them from the stress generated by exposure to environmental pollutants. Thus, the aim of this study was to analyze the effect of the fipronil insecticide on the GABAergic responses in the brain of Nile tilapia (*Oreochromis niloticus*) exposed to normoxia and hypoxia. This study seeks to better understand if fipronil interferes in the brain protection mechanisms of GABA and if the expression of antioxidant enzymes is modulated with changes in the levels of this receptor.

2. Methods

2.1 Animals and housing

Adult males (*O. niloticus*) of similar length and weight (12.92 ± 0.93 cm e 67.34 ± 11.62 g) were obtained from a fish aquaculture facility maintained in the State University of São Paulo - São José do Rio Preto, Brazil. Fish were first acclimated in tanks with capacity of 500 L (ca. 1 fish 5 L^{-1}), containing external filters and constant aeration at 27ºC, and 12:12D photoperiod (Boscolo et al., 2017) and remained for 30 days before the experiments began. The fish were fed twice a day to satiation with a commercial food (Guabi-Pirá, Brazil). All procedures were submitted and approved by the Committee for Ethics on Using Animal (CEUA), UNESP, São José do Rio Preto, SP, Brazil (protocol number 120/2015).

2.2 Experimental Design

2.2.1 Exposures

After the acclimation period the fish were placed individually (real replicas) in aquariums (17x10x10 cm) containing 17 L of dechlorinated tapwater and divided into 12 groups of five aquariums each (one fish per aquarium; *N* = 5; 60 aquariums). Six of the 12 aquariums were kept under constant aeration (dissolved oxygen > 6.0 ± 0.5 mg L^{-1}; Normoxia group), while the other six aquariums were subjected to hypoxia (≤ 2.0 ± 0.5 mg. L^{-1}; Hypoxia group) during the experimental period. Each of these two main groups, normoxia and hypoxia, were then subdivided into three subgroups of 10 aquariums that were exposed to fipronil at 0.1 and 0.5 µg.L^{-1} and one group that were not exposed to the contaminant (control group). After 3 and 8 hours of normoxia and hypoxia, in the presence or the absence of fipronil, five fish were collected and had their brains excised and immediately frozen in liquid nitrogen, for posterior analyses.

For hypoxia exposure, a system of specific connectors and hoses were coupled to the aquariums to allow the pumping of nitrogen gas (99% purity) into the water in order to maintain reduced levels of dissolved oxygen (≤ 2.0 ± 0.5 mg. L^{-1}). The dissolved oxygen concentration was measured at every hour to observe the variations during the experimental period, using an oxymeter HI-9146-04 (HANNA, Brazil) (Table 1). The exposure time were started after, approximately, one hour of pumping nitrogen gas in the water, after reaching the desired O_2 concentration, and the fish were added just after stabilization of oxygen concentration. Throughout the exposure time, the pumping of gaseous nitrogen into the water occurred only to keep the oxygen concentration constant. To avoid the access of the fish to air in the water/air interface, the water from hypoxic aquariums were covered with plastic film. For Fipronil exposure, Nile tilapias were exposed to nominal concentrations of fipronil (0.1 e 0.5 µg.L^{-1}), using the commercial REGENT® 800WG (800 g / Kg – 80.0 % m/v) insecticide. Concentrations of fipronil were based in studies reporting possible concentrations in surface waters that may be available in aquatic habitat, close to the agricultural regions in California/USA and Brazil (Tingle et al. 2003; Silva et al., 2009). The fish were exposed to each concentration in the presence and absence of moderate hypoxia for 3 and 8 hours. The animals were not fed, and no significant alterations was observed in dissolved O_2 for normoxia (5.66 ± 0.18 mg.L^{-1})and hypoxia (1.86 ± 0.18 mg.L^{-1}) conditions, and unionized ammonia ((0.09 ± 0.05 mg.L^{-1}) among the experiments groups, during the periods of exposure.

At the end of each exposure time, the animals were collected and anesthetized by immersion in water containing benzocaine (28 $mg.L^{-1}$ dissolved in ethanol), and then they were euthanized by cervical section for collection of the brain. For mRNA analysis, the brains were stored in RNA later, for stabilization and protection of RNA with immediate RNase inactivation. For the analyses of GABA concentrations, the brains were immediately frozen in liquid nitrogen, and all samples kept frozen at -80ºC until the analysis.

2.3 Quantitative real-time PCR analysis

2.3.1 Total RNA extraction and cDNA synthesis by reverse transcription

Total RNA was extracted from the brain samples (~50 mg) of the five individuals from each experimental group (N = 5) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and purity in each sample were measured using a spectrophotometer NanoDrop®ND-1000 (Thermo Scientific). Reverse transcription was performed using the QuantiTec® Reverse Transcription Kit (Qiagen) with 1 µg of total RNA. The complementary DNA (cDNA) was also quantified using the same spectrophotometer and the samples were diluted with ~10 µL of nuclease-free water (Qiagen). The aliquots of diluted cDNA were stored at −20ºC.

2.3.2 Primers design and real time quantitative PCR analysis

The complete and/or partial sequences of messenger RNA (mRNA) of each gene were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The selected genes of interest were: Receptors *GABA a-α, GABA a-β, GABA A-ү, GABA B1, GABA B2, GABA c-Rho,* hypoxia inducible factor (*HIF-1A*), antioxidant enzymes *SOD* and *CAT,* and Housekeeping genes β-actina and EF1α. The primers were designed based on the complete or partial mRNA sequences using OligoAnalyzer® e PrimerQuest® (IDT, http://www.idtdna.com) software’s. The primer sequences are described in Table 2.

Table 2. Primer sequences used for the qPCR for analyses of target and reference genes.

Gene name	Subunit	Primer F (5'-3')	Primer R (5'-3')
GABA A	ALFA (α)	TCCAAGTGTCTTTCTGGCTCAACA	GGCACTGATACTCAGCGTGGTCATA
GABA A	BETA (β)	CAACGAGGTGGGAGGAAACGAGATAA	CTGTACTGGATAGCCGAGTTGTCGAAG
GABA A	GAMA (γ)	GAGCGATGACGATGATGAGGTGTCT	TCACTGTTGGCTTGACTCCGATGT
GABA B	B1	GCCAGCGTTTCCCGACATTCTT	GCGATACGAGTCCACTTCCACTTCTT
GABA B	B2	CTCCAGAGTCTTCTGCTGTGCGTATAAC	TGTTCCCACCAGTTGCCCTGATA
GABA C	Rho	TTGGAGTGGATGTTCAGGTGGAGAG	TCTTGTTGGTGCTGCTGGTGAAG
HIF	-	ACCTTACCAAGACCCACCACAACT	ACCCACACGAAGCCTCCTTTCTTA
SOD	Enzyme	CGTGACAACACAGGTTGCTCTCC	CCTCCATTAGCTCTCCTTGTGGTTCA
CAT	Enzyme	CACTGAAGATGGTAACTGGGACCTGAC	TGGGAATGAACGAAGGATGGGAACA
β-actina	Housekeeping gene	CCGTTCATGAGTCGGCATAA	CCCTCCCACTGTTGCTTAAT
EF1α	Housekeeping gene	CGGTGTCATCAAGTCCGTTATC	GCATAAGCCATGCCTTGAGTATAG

Real time reactions were performed using 100 ng of cDNA samples per reaction, following the QuantiNova® SYBR® Green PCR kit methodology (Qiagen), in a Rotor-Gene TM 6000 thermocycler (Qiagen). Cycling conditions were: 2 min at 95 °C (activation) followed by 40 cycles of 5 s at 95 °C (denaturation) and 10 s at 60°C (annealing and extension). The PCR product was subjected to melting curve analysis to verify the specificity of the products formed for each gene. qPCR efficiency (E) was determined for each primer pair and checked by running a cDNA calibration curve. Relative transcription levels for each gene were analyzed using an efficiency corrected method ($2^{-\Delta Cq}$) and normalized by the geometric mean of reference genes (Schmittgen & Livak, 2008).

2.5 Quantification of GABA levels

GABA levels were analyzed in the fish brain following the method described by Van Ginneken et al., (1996), with modifications. Brain samples were individually weighted (20 mg) and homogenized in 0.08 mL of 0.5 M perchloric acid (PCA). The obtained mixture was maintained for 10 minutes at 4 °C and then centrifuged for 20 min at 15.000 *g* at the same temperature. Supernatants were collected and transferred to a tube containing potassium phosphate ice-cold (0.25 M KPi, pH 7.0) and centrifuged (20 min at 15.000 *g*). For amino acid quantification, the samples were derivatized with solution containing 7.2 mL methanolic OPA (5 $mg.mL^{-1}$), 2.2 mL 0.2

M borate buffer, pH 9.9 and 10 µL 3-mercaptopropionic acid (MPA). The sample and derivatizing solution ratio were 1:3, not exceeding 150 µL of total volume. The GABA levels were obtained injecting 10 μL^{-1} into the High-Performance Liquid Chromatography – HPLC (Shimadzu Corporation, Kyoto, Japan), consisting of one CBM20A communication bus module, two LC20AD-XR pumps, one DGU20A3R degassing unit, one SIL20AC-XR autosampler, and one CTO20AR column oven. The components were separated in an ACE C18 column (250 × 4.6 mm, 5 µm), coupled to a fluorescence detector set at 256 and 210 nm. The flow rate was 1.0 $mL.min^{-1}$ and the mobile phase consisted of 65% 60 mM K_2HPO_4, 30 µM acetic acid, 0.36 mM EDTA, adjusted to pH 7.0 (Pump A) and 35% methanol (Pump B). GABA was identified by its characteristic retention time of 7.5 min, as we determined by injections of authentic standard and construction of analytic curve for quantification.

2.6 Statistical Analysis

Statistical analysis was performed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA), while the graphics were made using GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA). The data normality was assessed using Normal Probability Plots of Residuals, some data were transformed into $\log_{10}$, when appropriated. For comparison analysis, we adopted General Linear Models (GLMs) with fatorial ANOVA design followed by Tukey's *post hoc* test, because this approach allows evaluating the effects of different conditions (normoxia or hypoxia), and of fipronil and exposure period, as well as any interaction between these predictors on the biomarkers evaluated. All gene transcript levels were expressed as relative fold change to the group that were not exposed to fipronil neither hypoxia (F0 group). The results were presented as mean ± standard deviation (SD), and we have considered $p < 0.05$ as statistical significant (Quinn and Keough, 2002; McDonald, 2014).

3. Results

3.1 Effects on gene transcription levels in brain

In general, the transcript levels of GABA receptors were statistically decreased or presented a decrease pattern in most of experimental groups after 3 hours of exposure

to fipronil, both in normoxia or hypoxia, but we did not observe significative differences in transcript levels of GABA-a (α) between the experimental groups (Fig.1).

The groups exposed to fipronil at the 0.1 and 0.5 µg.L^{-1} concentrations for 3 hours in normoxia showed a decrease (68 and 75%, respectively) in the GABA-a (β) transcript level compared to the group without fipronil (F0). However, the groups exposed to fipronil in the both concentrations during 3 hours of hypoxia, did not present significant differences between the treatments. Besides that, we observed a marked decrease effect (80%) of hypoxia on GABA-a (β) transcript levels regardless any fipronil treatment or time influences (Fig. 1B).

For GABA-a (γ), the transcript levels of the group exposed to fipronil 0.5 µg.L^{-1} decreased by 68% in relation to the group without fipronil and for 3 hours in normoxia. We also observed lower transcript levels (58 and 73%, respectively) in the groups exposed to fipronil at the 0.1 µg.L^{-1} and 0.5 µg.L^{-1} concentrations with hypoxia exposure, when compared group without fipronil and for 3 hours in normoxia (Fig. 1C).

The transcript levels of GABA-B2 receptor did not show significant influences of fipronil or hypoxia exposures. On the other hand, the combined effect of hypoxia + fipronil at both concentrations caused a ~80% decrease in the transcript levels of this gene, in relation to the subgroup under normoxia without fipronil exposure (Fig. 1E).

For the GABA-c receptor, only the subgroup exposed to fipronil at 0.5 µg.L^{-1} for 3 hours in hypoxia showed a 41% decrease in transcript levels, when compared to the group in normoxia without fipronil (Fig. 1F).

After 8 hours, we just observed statistical differences in transcript levels of GABA-B1 receptor. Only the combined effect (hypoxia + fipronil) at the highest fipronil concentration was capable of decreasing the transcript levels compared to all the other subgroups. (Fig. 1D).

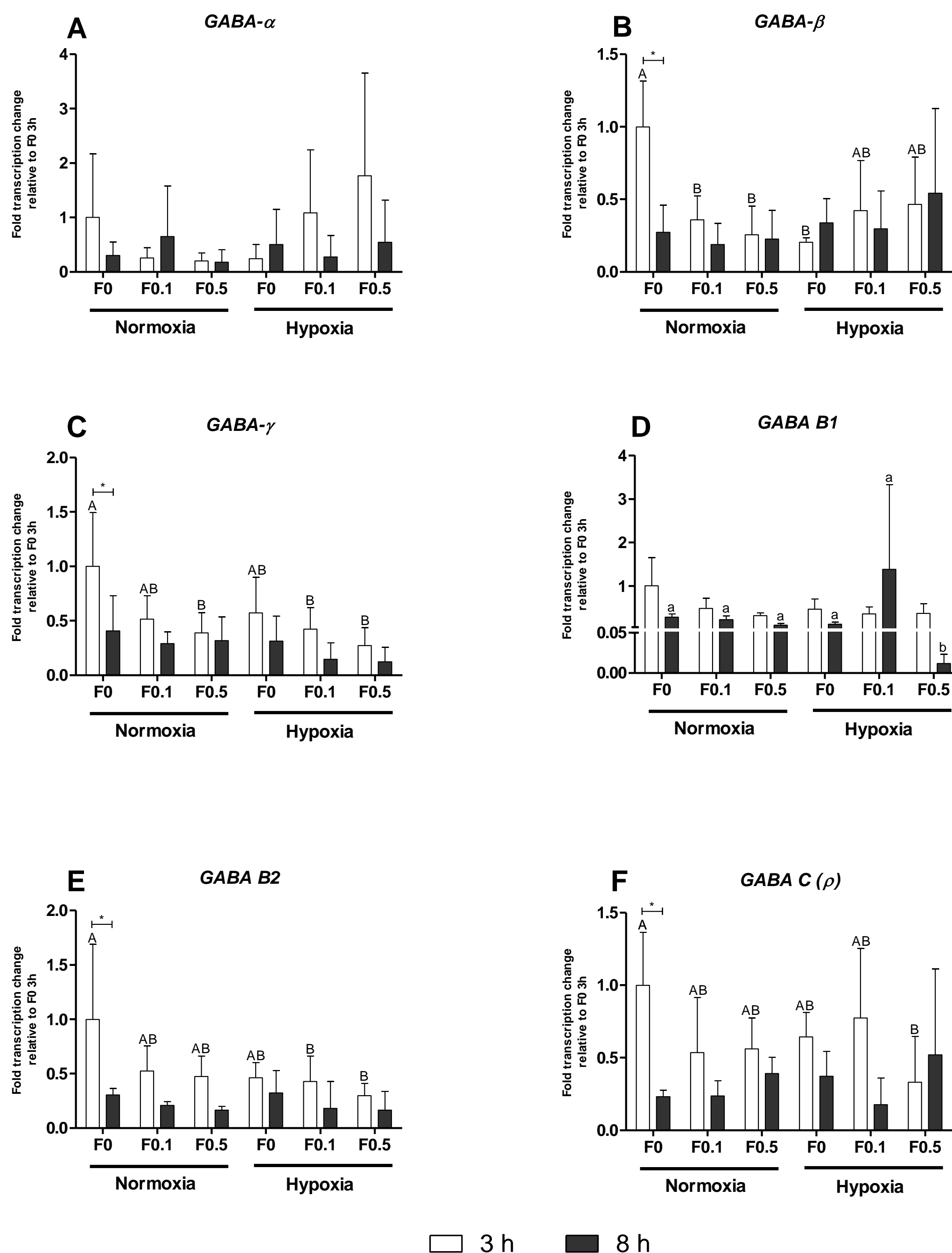

Figure 1. Normalized relative transcription ratio of **A** - GABA A (α), **B** - GABA A (β), **C**- GABA A- (γ) **D** - GABA B1, **E** - GABA B2, **F** - GABA C in brain of *O. niloticus* exposed to normoxia and hypoxia, at the 0.0, 0.1 and 0.5 ug.L^{-1} concentrations of fipronil, for 3 and 8 hours. The gene transcript levels were assessed by qRT-PCR and data are expressed as relative fold change to fipronil 0 group (F0). Different letters indicate statistical difference ($p<0.05$) between groups within each time of exposure. Horizontal bars indicate statistical difference ($p<0.05$) between exposure times.

During normoxia, in the group exposed to fipronil at 0.5 μg.L^{-1} for 3 hours, we observed a 66% decrease in transcript levels of the HIF-1A (α) compared to the group

without fipronil. In the groups exposed to hypoxia, we did not observe significant differences between the groups. However, a decrease by 76 and 83%, respectively, was observed in the groups exposed to fipronil at the both concentrations (0.1 and 0.5 µg.L^{-1}), relative to group without fipronil in normoxia (Fig. 2). The exposure to hypoxia for 3 hours caused a decrease in transcript levels by 66%, compared to the normoxia period. We did not observe significant differences in the groups exposed to normoxia and hypoxia during the 8 hours period.

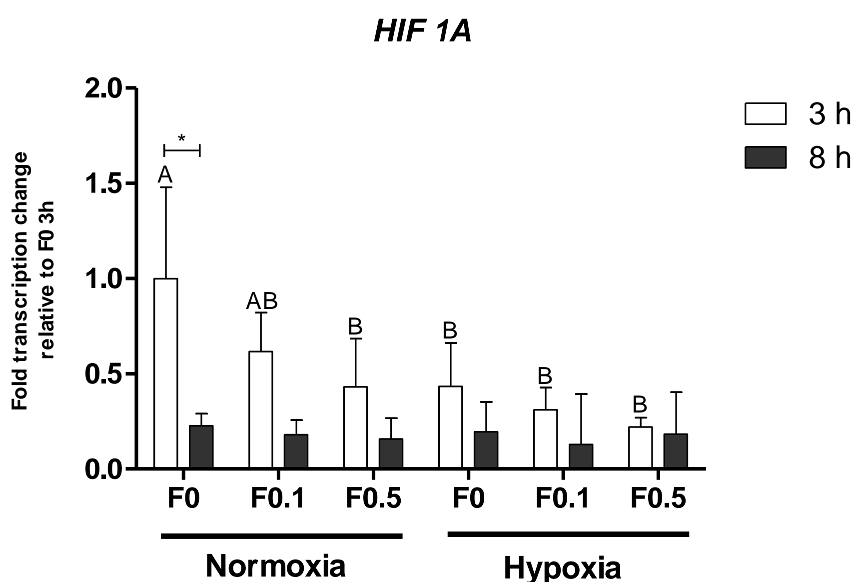

Figure 2. HIF 1A (α) gene transcription profiles in brain of *O. niloticus* exposed to normoxia and hypoxia, at the 0.0, 0.1 and 0.5 ug.L^{-1} concentrations of fipronil, for 3 and 8 hours. The gene transcript levels were assessed by qRT-PCR and data are expressed relative fold change to fipronil 0 group (F0). Different letters indicate statistical difference ($p < 0.05$) between groups within each time of exposure. Horizontal bars indicate statistical difference ($p<0.05$) between exposure times.

The exposure to normoxia or hypoxia did not alter *SOD* gene transcription, but the hypoxia exposure for 3 hours caused a decrease in transcript levels by 52% in the group exposed to fipronil (0.5 µg.L^{-1}), when compared to the normoxia group without fipronil in the CAT transcript levels (Fig. 3).

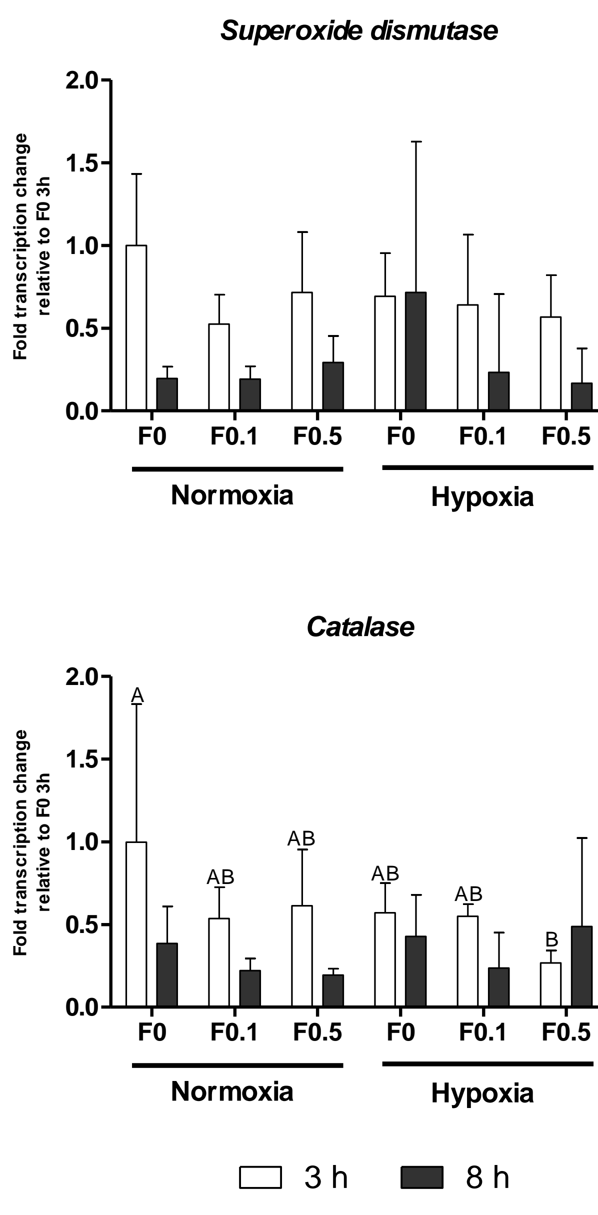

Figure 3. Normalized relative transcription ratio of the antioxidant enzymes genes Catalase and Superoxide Dismutase in brain of *O. niloticus* exposed to normoxia and hypoxia, at the 0.0, 0.1 and 0.5 ug.L^{-1} concentrations of fipronil, for 3 and 8 hours. The gene transcript levels were assessed by qRT-PCR and data are expressed as relative fold change to fipronil 0 group (F0). Different letters indicate statistical difference ($p < 0.05$) between groups within each time of exposure.

3.2 Effects on GABA neurotransmitter

Fipronil treatment, regardless its concentration, did not cause any alterations on brain GABA levels in the exposed fish, at both exposure periods. On the other hand, we could observe an interesting and solely hypoxia effect; it caused a ~3-fold decrease in GABA levels when compared to all other subgroups after 3h of experiment (Fig. 4). Intriguingly, regarding

the subgroup under the same experimental conditions (hypoxia without fipronil) but after 8h of experiment, the fish seemed to be able to restore GABA levels (Fig. 4).

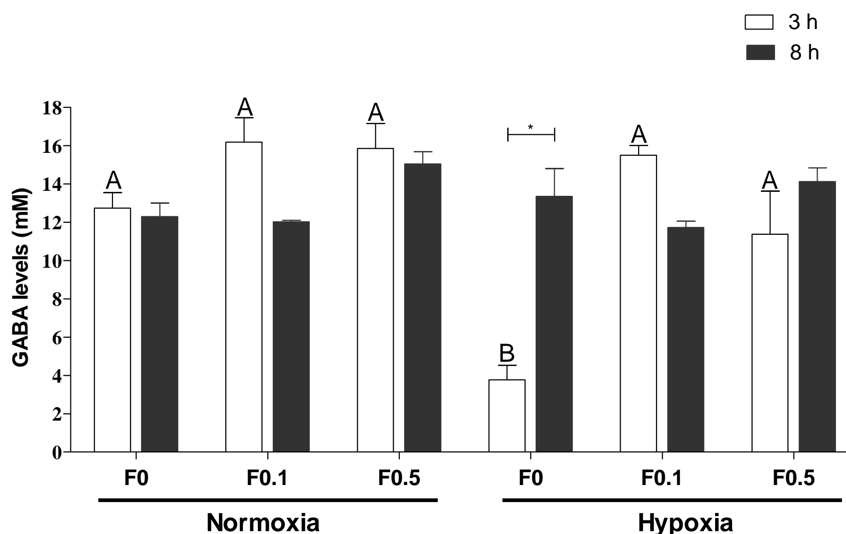

Figure 4. GABA levels in brain of de *O. niloticus* exposed to normoxia and hypoxia, at the 0.0, 0.1 and 0.5 ug.L^{-1} concentrations of fipronil, for 3 and 8 hours. Different letters indicate statistical difference ($p<0.05$) between groups. Horizontal bars indicate statistical difference ($p<0.05$) between exposure times.

4. Discussion

GABA plays a fundamental role in the regulation of the metabolism against negative effects of hypoxia (Lutz e Nilsson, 1997), mainly by decreasing the neuronal activity due to the GABA inhibitory action (Rubakhin et al., 1996). Therefore, we were interested to know if hypoxic condition in Nile tilapia would increase the transcription of receptors and GABA levels, in order to increase brain's protection against hypoxia energetic costs, and how the GABA receptor antagonist fipronil would interfere on these responses. It is known that in hypoxia situation, the animals increase, systematically, the GABA levels in the nervous system, in order to regulation of responsiveness and excitability neuronal in conditions of low oxygen (Kumar et al., 2011). However, unexpectedly, in this study hypoxia caused a significant decrease in the transcript levels of β subunit of GABA-a and HIF gene, and also caused a marked decrease in the brain GABA levels, after 3 h.

The GABA-a receptors are involved in the regulation of the neuronal action by controlling chlorine cellular influx, which can decrease resting membrane potential (Johnston, 1996). Decrease in GABA receptor transcription has been described as a

desensitization response due to GABA excess (Overstreet et al., 2000; Chang et al., 2002). Thus, we could hypothesize that the decrease in the transcript level of β subunit of GABA-a would be due to an excessive GABA release in response to hypoxia. However, these hypotheses are not supported by the observed decreased GABA levels observed in the fish submitted to hypoxia, suggesting a decrease in GABAergic neurotransmission in tilapias under 3 h of hypoxia. We propouse to explain these results would be due to an excitatory activity of GABA in neurons containing excessive intracellular chlorine ions, as well documented in mammals (for a review, see Marty and Llano, 2005) and in aquatic invertebrates (Cheung et al., 2006) and vertebrates, including fish (Corda et al., 1989; Nakane and Oka, 2010; Wirbisky et al., 2014). In this case, decreased GABA neurotransmitter levels and GABA-a β subunit transcript levels would be a consequence of a general inhibitory effect on the nervous systems. However, this does not agree with previous findings on classical GABAergic responses to hypoxia in fish, in which GABA levels usually increases in brain under oxygen deprivation (Nilsson et al., 2004), an effect also previously observed in tilapias (Ginneken et al., 1996). Nevertheless, it should be noted that most studies on oxygen deprivation in fish are related to anoxic conditions. In this study, Nile tilapias were submitted to hypoxia, which represents a different situation considering that low oxygen levels were available for the fish (~2.0 $mg.L^{-1}$). Therefore, we suggest that the observed decrease in GABA concentrations and on transcript levels of the β subunit of GABA-a receptor during the first 3 h of hypoxia may be related to a mild decrease in the oxygen concentrations. As the tilapias are highly resistant to variations in oxygen availability, they would not require such rapid up-regulation of the GABAergic system to decrease metabolism in the brain, when oxygen concentration is not so limited. The GABAergic system plays an essential role in the temporal control of neuronal activity, and for this reason, the timing of receptor activation is important, and GABA levels in the extracellular compartment must be carefully regulated (Scimemi et al., 2014). In this study, Nile tilapias were submitted to hypoxia, which represents a different situation compared to anoxia, considering that low oxygen dissolved levels were available for the fish (~2.0 $mg.L^{-1}$). Thus, we suggest that in moderate hypoxia, to supply the lack of oxygen, initially the animal increases the ventilation rate of the gills and, hence, an excitatory response occurs in the central nervous system, mediated by

glutamate (Cheung et al., 2006). Taking into consideration that GABA and glutamate synthesis involve common precursors, since the glutamate undergoes decarboxylation generating GABA, it can be suggested that in the first three hours of oxygen deprivation a deviation occurs in the GABA synthesis to increase the glutamate, increasing the neurons excitability involved in the ventilation. Moreover, the previous findings on classical GABAergic responses to hypoxia in fish indicate that GABA levels usually increase in brain under oxygen deprivation (Nilsson et al., 2004), an effect also previously observed in tilapias (Ginneken et al., 1996), it should be noted that most studies on oxygen deprivation in fish are related to anoxic conditions. However, this hypothesis needs to be better clarified, although it has already been documented in hypoglycemia situations (Madl e Royer, 2000).

Besides our study demonstrated a decrease in the GABA and GABA-a β in the initial periods of hypoxia exposure, after 8 h of hypoxia, the levels of this neurotransmitter and GABA receptor increased to control levels after 8 h, possibly indicating an initial brain response to increase GABAergic activity as a protective mechanism to prolonged hypoxia. Despite not higher than normoxic fish after 8 h, GABA levels would increase after longer periods of hypoxia, but this remains to be further clarified. Similarly, GABA receptors and GABA levels were also unchanged in the epaulette shark (*Hemiscyllium ocellatum*) submitted to hypoxia (not anoxia) for 12 hours (Mulvey and Renshaw, 2009).

Fish exposed to fipronil for 3 h presented decreased transcript levels of the β and γ subunits of GABA-a receptor, clearly demonstrating an impairment effect of fipronil on the GABAergic neurotransmission. The binding of fipronil to fish GABA-a receptors was previously demonstrated by Zhang et al. (2018) in the bighead carp (*Aristichthys nobilis*). These authors demonstrated that fipronil has high affinity to GABA-a receptors of the fish, similar to the affinity found on insects, suggesting fipronil as highly toxic to fish. Considering the presented information, it can be suggested in this study that, by binding on GABA-a receptors, fipronil impaired the opening of chlorine channels, suppressing the GABA-mediated inhibitory effect on brain. The decrease on mRNA levels of GABA-a receptors (subunits β and γ) after 3 h could be due to a desensitization of the receptor because to excessive fipronil binding, or another hypothesis is that the fipronil inhibits the receptor by promoting entry into a

distinct, long-lived non-conducting, that is, blocked state, mechanisms that proceed independently, such as suggest by Lii & Akk (2008). The exact mechanisms that lead to GABA-a receptor desensitization or blocked state of receptors is not fully known, but the process is associated with a decline in the frequency of channel openings rather than with a change in open intervals (Hamill et al., 1983; Weiss, 1988). In the other hand, the unaltered GABA levels observed in the groups exposed to fipronil for 3 h, also reforce the ideia that fipronil modifies receptor behaviour by introducing a novel blocked receptor state, since it has been documented that receptors driven into the inhibited state are incapable of undergoing desensitization (Lii & Akk, 2008) which may have contributed by not altering GABA levels in these groups. After 8 h, fipronil did not cause any effect on GABA levels nor GABA receptor transcript levels, probably due to its elimination from brain, thereby allowing the adjustment of GABAergic system.Therefore, it is interesting to note that 3 h of hypoxia caused a marked decrease in GABA levels, and the fipronil exposure under hypoxia for 3 h prevented a decrease in GABA levels compared to hypoxia alone. When combined fipronil exposure with hypoxia, GABA levels were significantly higher than the levels observed in fish only submitted to hypoxia, which clearly demonstrates the effects of fipronil. As discussed, we proposed that initial periods of hypoxia caused an excitatory response of the nervous system in order to increase ventilation, thus causing a decrease in GABA concentration. In contrary, fipronil exposure increased GABA levels, possibly due to the blockade of GABA receptors. When combined to hypoxia, fipronil effects were prevalent, leading GABA levels to increase as a response to receptor blockade. This result also demonstrates the interference of fipronil on GABAergic responses to hypoxia, in accordance to our hypotheses.

The exposure to fipronil combined with hypoxia for 3 h caused a significant decrease in the transcript levels of the subunit γ of GABA-a receptor, and in the transcription of GABA-B2 and GABA-C (ρ) receptors. The effect on the subunit γ of GABA-a receptor would be due a fipronil effect. As discussed, fish exposed to fipronil presented low levels of this transcript, while fish exposed only to hypoxia did not. However, only fish exposed to the highest fipronil concentration presented lower GABA-a (γ) transcript levels, while fipronil at both concentrations caused the same effect under hypoxia, evidencing the contribution of hypoxia to the fipronil effects on

this receptor. More important, the combined effect of fipronil and hypoxia on the transcript levels of GABA-a (γ), GABA-B2 and GABA-C (ρ) receptors reveals that fipronil alters the GABAergic responses to initial periods of hypoxia in tilapia's brain. Indeed, after 8 h, a marked decrease in the transcript levels of GABA-B1 was also observed in the fish exposed to the higher fipronil concentration combined to hypoxia, corroborating the impairment of the GABAergic neurotransmission caused by this insecticide during hypoxia.

HIF-1 is a transcription factor consisted by an alpha subunit and a beta subunit. The beta subunit is constitutively expressed while the alpha subunit is inducible, which acts on the regulation of expression of numerous genes involved in the maintenance of cellular homeostasis during oxygen scarcity (Chandel et al., 1998; Lee et al., 2004. Compared to normoxia values, we observed an overall decrease in HIF transcript levels in all the experimental groups after 3 h, except in the group exposed to the lower concentration of fipronil under normoxia. Although HIF is expected to increase under hypoxia conditions, it has already been discussed that this regulation is dependent on the evaluated organ, fish species and hypoxia condition (Rimoldi et al., 2012; Li et al., 2017). Also, in accordance to our hypothesis, the initial hypoxia periods would have an excitatory effect on nervous system, leading to increase ventilation, therefore causing a decrease in HIF, even in the presence of fipronil. The decrease in HIF transcript levels after 3 h of exposure to the higher fipronil concentration without hypoxia would also indicate a higher oxygen consumption to deal with fipronil desintoxication. The response of HIF-1A to hypoxia situation have been of the transient nature, once that some studies have shown increased HIF transcript levels (Agani et al., 2000; BelAiba et al., 2007), other studies have demonstrated a decrease in HIF1α (Wartenberg et al., 2003; Callapina et al., 2005), and some shown no effects on transcript levels (Vaux et al., 2001), thus, in general, the informations about the HIF transcript levels are conflicting, and moreover, the HIF expression, activation, and function can be influenced for various cellular and tissue stress response, including ROS production.

Another hypothesis to explain the decrease in HIF-1A transcript levels is the moderate hypoxia (2.0 $mg.L^{-1}$) and relative long time of exposure (3h and 8h) that fish were submitted. BelAiba et al., (2007) suggested that hypoxia initiates a rapid but

transient increase of HIF-1A mRNA that occurs with a maximum at 1 h returning to basal levels within 4 h, in a process that involves activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and of nuclear factor-κB (NFκB), which in turn binds and activates the HIF-1α. This effect was also observed by Wang et al., (1995) that showed a transient increase in response to hypoxia with a first peak at 1–2 h and a subsequent decline to basal levels within 8 h, corroborating our results after 8 h. Besides that, it should be mentioned that the most part of studies that observed increases in the HIF mRNA levels were done on animals exposed to 7% (Wiener et al., 1996), 1% (Turcotte et al., 2003), or 0% O_2 (Yu et al., 1998).

The effects of fipronil and hypoxia were also evident in the gene transcription of antioxidant enzyme CAT, that was significantly lower in those fish exposed for 3 h to the combination of hypoxia and the higher concentration of fipronil, though the SOD mRNA levels were unchanged among experimental groups. This result indicates the impairment of antioxidant defenses caused by fipronil during hypoxia periods, turning fish more susceptible to oxidative stress. It is known that HIF activation might prevent excessive ROS production in hypoxic cells by regulating mitochondrial respiration through increased expression of PDK1 and switching of cytochrome c oxidase subunit 4 isoform 14 (Saito et al., 2015). Thus, the results of CAT mRNA and decrease in HIF-1A mRNA suggests a mitigating effect of fipronil (0.5 $\mu g.L^{-1}$) during hypoxia, because we did not observe any difference between the other treatments. Therefore, the decrease in CAT transcript can suggest a increase in ROS concentrations, that did not have a considerable effect for direct HIF-1A activation during hypoxia, probably because the HIF-1A signaling can be dependent on some reactive oxygen species, and not others (Brunelle et al., 2005). On the other hand, as proposed by Qutub & Polel (2008), the HIF-1A regulation during hypoxia, that occurs in less than 3 hours, provides a response within minutes (HIF-1A accumulation during hypoxia), thus, as the oxygen is restored in few hours, there is no necessary to keep the response for long time, thus, the duration the cell response to stress is insufficient to trigger the HIF-1A activation.

In conclusion, our results reveal different mechanisms by which fish survives to situations of hypoxia in the presence of the fipronil insecticide. We demonstrated that during the 3 h of exposure to hypoxia a decrease in GABAergic response was

observed, probably because of an initial excitatory response of nervous system to increase ventilation under low oxygen availability, which may have also contributed to the observed decreases in HIF transcript levels, even in the presence of fipronil. The fipronil exposure caused a blockade of GABA-A receptors, that caused a desensitization of GABA-A receptors, leading to a downregulation of its transcription. Fipronil exposure for 3 h altered the fish response to hypoxia, decreasing CAT mRNA levels and causing changes in the transcript levels of GABA-A (β and γ subunits), GABA-B2 and GABA-C (ρ) receptors, when compared to the fish exposed only to hypoxia. Moreover, considering that the establishiment of social relations of hierarchy and dominance in tilapia populations are often related to the GABA inhibitory action, the changes observed in this study suggest that the presence of agrochemicals in combination with variationsin oxygen availability may affect the natural behavior of the fish, which in the long run may cause significant problems in mortality, hampering community structure and ecosystem.

Conflict of interest statement

Authors do not have any potential conflict of interest relevant to this article.

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CAPÍTULO II

Genotoxic and oxidative stress markers in *Oreochromis niloticus* after isolated and combined exposure to hypoxia and fipronil.

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Abstract

The presence of chemical contaminants in aquatic environmental, such as fipronil, can influence the develop adaptation mechanisms of fish to survive in hypoxia situations.The purpose of this study was evaluating fipronil effects on oxidative stress markers in gill and liver of Nile Tilapia submitted or not to hypoxia, in order to better understand fipronil interferences in fish response mechanism to hypoxia. *O. niloticus* were exposed for 3 and 8 hours to hypoxia and fipronil (0.1 e 0.5 µg L^{-1}), isolated or in combination. Low dissolved oxygen (<2 mg/L^{-1}) in the water was maintained by pumping gaseous nitrogen in water. After eachp period of exposure, we evaluated the activity of the enzymes catalase (CAT), glutathione *S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and lipid peroxidation levels were evaluated in the gills and liver, along with genotoxic damage in the erythrocytes. Our results demonstrated that hypoxia as an environmental stressor may modulate biochemical responses in exposed organisms, making the animals more susceptible to oxidative damage. The antioxidant defense systems of the fish remained elevated during the first hours of exposure, possibly to adapt to stress conditions. In the groups exposed to fipronil, concentrations and times influenced the antioxidant defense system, altering the activity of the CAT, GR antioxidant enzymes, and th conjugation enzyme GST. Besides that, we observed that the mechanisms of detoxification are specific for each tissue and the most significant changes were found in the gills. The presence of combined stressors has been shown to cause greater disturbances in biochemical responses when compared to individual factors, as well as to induce genotoxic damage and lipid peroxidation in fish tissues.

Palavras-Chave: Fish, Antioxidant enzymes, Lipid peroxidation, Oxidative stress, Comet assay

1. Introduction

Aquatic environments are continuously affected by changes in environmental factors, especially abiotic factors such as pH, temperature, chemical contamination and variations in oxygen availability (Sampaio et al., 2012). In most tropical aquatic environments, it is common that excessive decomposition of organic matter in association with high temperatures cause depletion of dissolved oxygen (DO), a situation known as environmental hypoxia (Sampaio et al., 2008). Environmental hypoxia often occurs when DO in water is less than 2 $mg.L^{-1}$, which is critical for several aquatic vertebrates, such as fish. To survive under environmental hypoxia, hypoxia-tolerant fishes have developed adptative mechanisms that allow them to decrease the aerobic energy consumption and to increase anaerobic ATP production (Tiedke et al., 2014). This ability to decelerate pathways of high energy consumption is a key strategy for survival, which might include alterations in transcription and/or translation rates of key metabolic genes, changes in the activity and expression of kinases and phosphatases involved in cellular signaling, and modulation of transcription factors and microRNAs (Storey and Storey, 2012).

Some hypotheses sustain the idea that the decrease in DO concentrations under hypoxic conditions may induce oxidative stress, through the decrease in the rate of electron transport chain, allowing electrons to escape and being intercepted by molecular oxygen (Waypa et al., 2016). This process causes the partial reduction of molecular oxygen generating highly reactive intermediate species, such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). Another mechanism by which oxidative stress takes place would be related to the xanthine reductase/xanthine oxidase system, since the first enzyme can be converted to the second one under hypoxia, increasing the generation of reactive oxygen species (ROS) (Hermes-Lima et. al., 2015). As a response to increased ROS, antioxidant defenses are stimulated to intercept ROS and to avoid oxidative damage, also preventing oxidative stress during reoxygenation period (Almeida e Di Mascio de 2011; Hermes-Lima et al., 2015). In accordance, several studies have reported alterations in antioxidant enzymes and levels of oxidative lesions in fish submitted to hypoxia (Lushchak et al. 2005; Zhao et al., 2015).

Although the efficacy of the mechanisms of adaptation to hypoxia is well developed in evolutionarily adapted organisms, they depend on the animal health state. In this context, metabolic changes due to environmental pollutants exposure may negatively affect the adaptive responses of fish to hypoxia. Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfonyl)-pyrazole-3-carbonitrile), is a phenyl-pyrazole insecticide. Has been one of the most used insecticides in the cultivation of sugarcane, cotton and corn in many countries. Its presence in aquatic compartments has often been detected in surface water and sediments (Schlenk et al., 2001; Sprague e Nowell, 2008; Harman-Fetcho et al. 2005). Fipronil toxic effects on non-target species have attracted greater attention because of its growing use in agriculture and veterinary medicine. Although higher doses of fipronil are required to induce more drastic effects such as mortality (LC50 for *Danio rerio* – 220.4 $\mu g.L^{-1}$ (Wu et al., (2014); *Oreochromis niloticus* - 0,25 $mg.L^{-1}$ (USEPA, 1996); *Poecilia reticulata* - 0.09 $mg.L^{-1}$ (Manrique et al., 2013), long-term sublethal effects to aquatic organisms have also been reported (Hayasaka et al., 2012), such as oxidative stress and genotoxic effects (Van Der Oost et al. 2003; Mustafa et al. 2011). Several studies have indicated that fipronil may induce oxidative stress in fish (Wang et al., 2016; Beggel et al., 2012; Wu et al., 2014), causing changes in the antioxidant defense system and thereby increasing the levels of damage to macromolecules such as lipids, DNA and proteins (Classen et al., 2012). In addition, a study conducted *in vitro* with *SH-SY5Y* human neuroblastoma cells, has suggested that fipronil is a strong uncoupler of oxidative phosphorylation, mediating increases in the generation of ROS at relatively low concentrations (Fipronil concentration 25-200µM) (Ki et al., 2012).

Despite the few studies related to the metabolism of fipronil, it is known that oxidation and reduction reactions, catalyzed by cytochrome P450 enzyme complex, especially CYP3A4, generate different degradation products (i.e. fipronil desulfinil and fipronil sulfone), which can be more toxic to insects, mammals, aquatic organisms and birds compared to the original compound (Leghait et al., 2009; Tavares et al., 2015). The metabolism of fipronil into its degradation products has also been pointed out as an activation mechanism for toxicity of the compound. In the bloodstream, fipronil and its metabolites are widely distributed throughout the body due to its

intense enterohepatic recirculation and high half-life, thus becoming an additional factor to its toxicity, potentializing its effects (DAS et al., 2006).

Studies about interactions between contaminant exposures with physiological protective responses against negative effects of hypoxia in aquatic animals are still scarce in the scientific literature. Considering that fipronil would instigate oxidative stress in animals, and that fish developed adaptive protection mechanisms against ROS generated during hypoxic situations, in this study we were interested to know how fipronil exposure would affect oxidative stress markers in the gill and in the liver of fish submitted to hypoxic conditions. We hypothesized that both fipronil and hypoxia increase the rate of ROS generation, causing DNA damage and lipid peroxidation, as well as adaptation of antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase and glutathione S-transferase). Moreover, such effects are substantially increased when fipronil exposure is combined with environmental hypoxia.

2.2 Experimental Design

2.2.1 Exposures

After the acclimation period the fish were placed individually in aquariums (35 x 25 x 15 cm) containing 17 L of tapwater and divided into 12 groups of five aquariums each (one fish per aquarium; $N = 5$; 60 aquariums). Six of the 12 aquariums were kept under constant aeration (dissolved oxygen > 6.0 ± 0.5 mg L^{-1}; Normoxia group), while the other six aquariums were subjected to hypoxia ($\leq 2.0 \pm 0.5$ mg. L^{-1}; Hypoxia group) during the experimental period. Each of these two main groups, Normoxia and Hypoxia, were then subdivided into three subgroups of 10 aquariums that were exposed to fipronil at 0, 0.1 and 0.5 $\mu g.L^{-1}$ of Fipronil (nominal concentration).

For hypoxia exposure, a system of specific connectors and hoses were coupled to the aquariums to allow the pumping of nitrogen gas (99% purity) into the water in order to maintain reduced levels of dissolved oxygen ($\leq 2.0 \pm 0.5$ mg. L^{-1}). The dissolved oxygen concentration was measured at every hour to observe the variations during the experimental period, using an oxymeter HI-9146-04 (HANNA, Brazil)

(Table 1). The exposure time were started after, approximately, one hour of pumping nitrogen gas in the water, after reaching the desired O_2 concentration, and the fish were added just after stabilization of oxygen concentration. Throughout the exposure time, the pumping of gaseous nitrogen into the water occurred only to keep the oxygen concentration constant. To avoid the access of the fish to air in the water/air interface, the water from hypoxic aquariums were covered with plastic film. For Fipronil exposure, Nile tilapias were exposed to nominal concentrations of fipronil (0.1 e 0.5 $\mu g.L^{-1}$), using the commercial REGENT® 800WG (800 g / Kg – 80.0 % m/v) insecticide. Concentrations of fipronil were based in studies reporting possible concentrations in surface waters that may be available in aquatic habitat, close to the agricultural regions in California/USA and Brazil (Tingle et al. 2003; Silva et al., 2009). The fish were exposed to each concentration in the presence and absence of moderate hypoxia for 3 and 8 hours. The animals were not fed, and the ammonia concentrations of water did not show significant differences during the periods of exposure.

After 3 and 8 hours of normoxia and hypoxia, in the presence or the absence of fipronil, five fish were collected of each treatments and anesthetized by immersion in water containing benzocaine (28 $mg.L^{-1}$ dissolved in ethanol), and then they were euthanized by cervical section for collection by cervical section for collection the gills and liver, which were frozen in liquid nitrogen and kept in freezer -80ºC until the analyses. The fish blood for comet analysis withdrawn by caudal puncture.

2.4 Analysis of antioxidant enzymes

For the evaluation of the antioxidant enzymes (CAT, GPx, GR, and SOD activities) and conjugation enzyme (GST activity) in the gills and livers of fish were homogenized (1:4; weight:volume) in 20 mM Tris-HCl, pH 7.5, containing sucrose 0.5 M, EDTA 1 mM, DTT 1 mM, 0.15 M KCl and 1 mM protease inhibitor (PMSF). After homogenization, samples were centrifuged for 20 minutes at 10,000 *g* at 4ºC. The supernatant fraction was collected and centrifuged again for 1h at 50,000 *g*. The second supernatant fraction was collected and stored at −80 °C for subsequent evaluations.

CAT activity was measured in a Thermo Evolution 300 spectrophotometer with a dual beam, according to the method described by Beutler (1975), which monitors the

rate of decomposition of hydrogen peroxide (H_2O_2) by the enzyme at 240 nm. The activities of GPx, GR e GST were performed on a Victor TM X3 microplate reader (Perkin Elmer®). GPx activity was measured following the method described by Sies et al. (1979), monitoring the decrease in absorbance at 340 nm as a result of NADPH consumption by GR to reduce GSSG produced due to GSH consumption by GPx in the reduction of the substrate *t*-butyl hydroperoxide (tBOOH). GR activity was measured using the method described by Carlberg e Mannervik (1985), monitoring the reduction of GSSG to GSH by the enzyme, which uses NADPH as electron donnor, causing a decrease in absorbance at 340 nm. GST activity was analyzed following the method described by Keen et al. (1976), monitoring the formation of the conjugate of the substrate 1-chloro-2,4-dinitrophenyl benzene with GSH, at 340 nm. SOD activity was measured using a SOD Assay Kit-WST (Sigma, Aldrich), following the protocol proposed by Freitas et al. (2017). Proteins were quantified in the samples using method proposed to Bradford (1976) at 595 nm, with bovine serum albumin as the standard.

2.6 Lipid Peroxidation

Lipid peroxidation was evaluated by measuring Malondialdehyde (MDA) levels through the "thiobarbituric acid reactive substances" (TBARS) assay. Gill and liver samples (100 mg) were homogenized in 0.3 mL of buffer Tris-HCl 0.1 M, pH 8.0. After homogenization, 300 µL of thiobarbituric acid (TBA, Sigma-Aldrich, Germany), diluted in HCl 0.2 M (0.4%) were added to the sample. The mixture was heated at 90ºC for 40 minutes and the reaction product was extracted with 1.0 mL of n-butanol. The malondialdehyde concentration (MDA) and 2-thiobarbituric acid (TBA) quantification was made by High Performance Liquid Chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan), coupled to the UV/Vis detector, according to the procedure described by Almeida et al. (2004). The MDA quantification was performed based on a standard curve, injecting autheintic MDA standards previously prepared using the same procedure as for the samples. The chromatographic column used in the analyzes was a Shimadzu C18 column (150 × 4.6 mm, 5 µm). The mobile phase used was 50 mM potassium phosphate solution at pH 7.0 (60%) and methanol (40%), and at a flow rate of 1.0 mL/min. Data were expressed as pmol MDA / mg tissue.

2.7 Comet assay

For genotoxic analysis, 10 μL of fish blood withdrawn by caudal puncture were diluted in 1000 μL of physiological solution. In previously gelatinized slides with common agarose, 10 μL of the cell suspension and 120 μL low melting point agarose (0.5%) at 37°C were added. The slides remained in lysis solution (1 mL triton X-100, 10 mL DMSO and 89 mL lysis stock solution, pH 10.0 – stock solution: NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, para 1 L) in the refrigerator for 24 hours prior to the start of the analysis.

Genotoxic damage to fish erythrocytes was assessed by means of an alkaline electrophoresis (pH ~13.0). After the processing of blood samples and cell lysis, the slides were transferred to a horizontal electrophoresis system for 20 minutes, in the light absence. After this period, the slides were submitted to electrophoresis for more 20 minutes at 25 V and 300 mA. Then, the slides were neutralized with Tris 0.4 M (pH 7.5), for 15 minutes fixed in ethanol for another 10 minutes. Two slides were prepared per sample, stained with 100 µL Ethidium Bromide (0.002 mg/mL), and a total of 100 nucleoids were analyzed (50 in each slide), using fluorescence microscopy, filter B – 34 (excitation: λ = 420 – 490 nm, barrier: λ = 520 nm), in 40x objective and the nucleoids evaluated using the Software Cometer IMAGEM 2.2 (META SYSTEMS). The variables analyzed were the tail length and the percentage of DNA in the tail.

2.9 Statistical Analysis

Data normality were assessed using Normal Probability Plots of Residuals. For comparisons among groups, we used General Linear Model (GLM), with two-way ANOVA design, followed by Fisher *post hoc* test. To evaluate any significant differences ($p<0.05$), we considered only treatment*time interaction among groups. Results are presented as mean ± standard deviation (SD). The graphics were done using GraphPad Prisma version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Antioxidant Enzymes

The activity of CAT was significantly decreased in the gills of fish exposed to 0.5 $\mu g.L^{-1}$ of fipronil for 3 h, and in the gills of fish submitted to hypoxia and fipronil at

0.1 and 0.5 µg.L^{-1} for 3 h (Fig. 1A), when compared to the control group at 3 h. After 8 h, no alteration was observed in CAT activity between experimental groups. GPx activity was significantly increased in the gill after 3 h of hypoxia exposure, and after 8 h of exposure to hypoxia and 0.5 µg.L^{-1} of fipronil, when compared to the respective control groups (Fig. 1B). GR activity was unchanged among experimental groups (Fig. 1C). Hypoxia exposure for 3 h in combination with firponil at 0.1 and 0.5 µg.L^{-1} caused a significant decrease in GST activity in the gills, compared to those fish exposed only to hypoxia (Fig. 1D). Hypoxia exposure for 8 h combined with exposure to fipronil at 0.1 and 0.5 µg.L^{-1} significantly decreased GST activity in the gills in comparison to the group exposed only to fipronil at 0.5 µg.L^{-1} or only to hypoxia (Fig. 1D).

In the liver, CAT activity was higher than controls in the groups exposed to hypoxia for 3 hours and hypoxia combined with fipronil in the two concentrations (Fig. 2A). After 8 h, CAT activity was significantly lower than controls in the liver of the fish from the groups exposed to fipronil at µg.L^{-1}, isolated or in combination with hypoxia. In the liver, the activity of GPx was unchanged (Fig. 2B), whereas GR activity was higher in the groups exposed to hypoxia combined with fipronil 0.1 µg.L^{-1} (3 h), and in the groups exposed only to hypoxia or only to fipronil 0.1 µg.L^{-1} (8 h), when compared to their respective control groups (Fig. 2C). Also, in the liver, the activity of GST was significantly higher in those fish exposed only to hypoxia (3 h) or exposed to fipronil at 0.5 µg.L^{-1}, isolated or in combination with hypoxia (8 h), compared to their respective control groups (Fig. 2D).

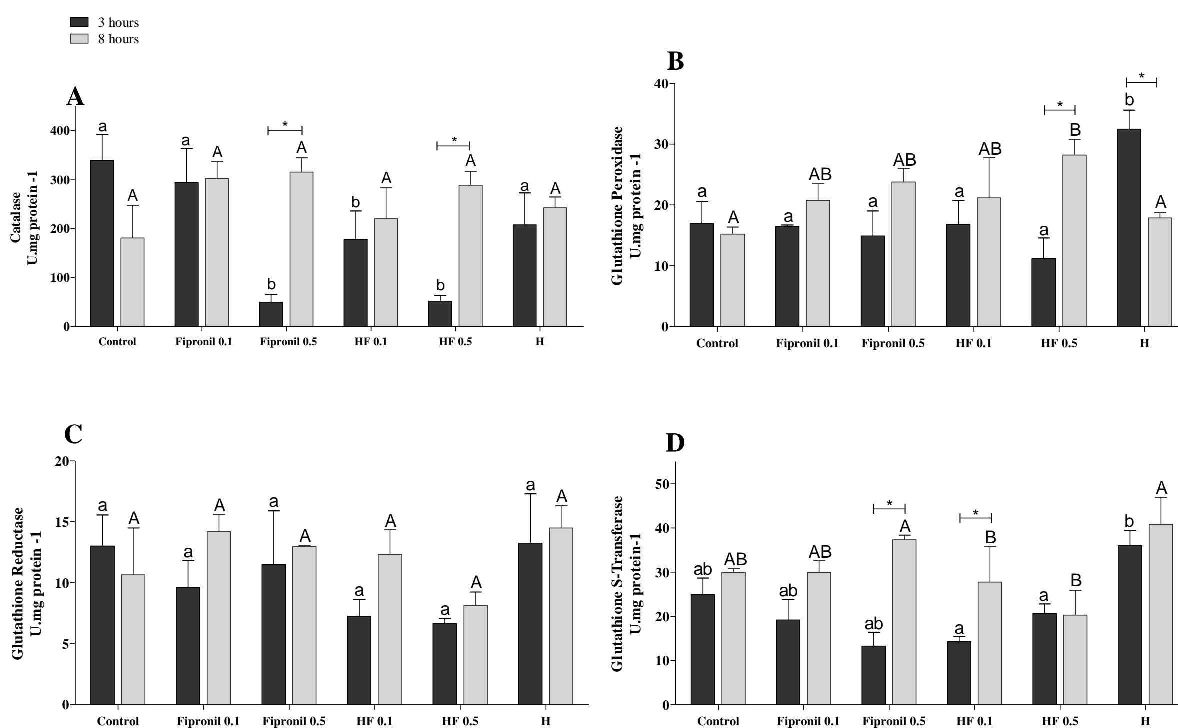

Fig. 1: Antioxidant enzymes activity A: Catalase, B: Glutathione Peroxidase, C: Glutathione Reductase and D: GutathioneS-transferase (GST) in the gills of O. niloticus exposure to fipronil (F 0.1 µg.L-1 e F 0.5 µg.L-1), Hypoxia (H) and Hypoxia with Fipronil (HF 0.1 µg.L-1 e HF 0.5 µg.L-1). Different lower letters indicate statistical difference between groups into the 3 hours period. Different capital letters indicate statistical difference between groups into the 8 hours period. Bars indicate statistical differenc between exposure times.

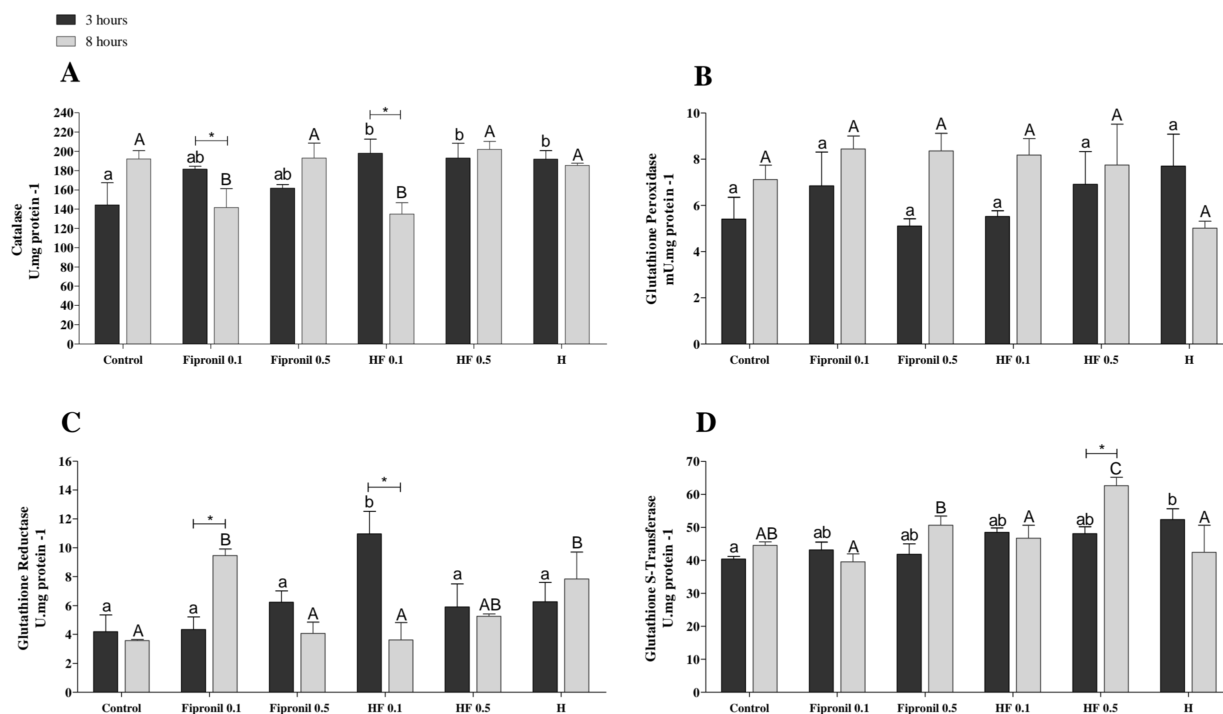

Fig. 2: Antioxidant enzymes activity **A:** Catalase, **B**: Glutathione Peroxidase, **C:** Glutathione Reductase and **D:** GutathioneS-transferase (GST) in the liver of *O. niloticus* exposure to fipronil (F 0.1 µg.L^{-1} e F 0.5 µg.L^{-1}), Hypoxia (H) and Hypoxia with Fipronil (HF 0.1 µg.L^{-1} e HF 0.5 µg.L^{-1}). Different lower letters indicate statistical difference between groups into the 3 hours period. Different capital letters indicate statistical difference between groups into the 8 hours period. Bars indicate statistical differenc between exposure times.

3.2 Lipid peroxidation

Lipid peroxidation levels were significantly lower in the gills of all experimental groups, compared to the control group, after both 3 and 8 h (Fig. 3A). In the liver, lipid peroxidation levels after 3 h were significantly increased in the fish exposed to fipronil 0.5 µg.L^{-1}, when compared to the control group and to the group subjected to hypoxia (Fig. 3B). After 8 h, no differences were observed in the levels of lipid peroxidation in the liver among experimental groups.

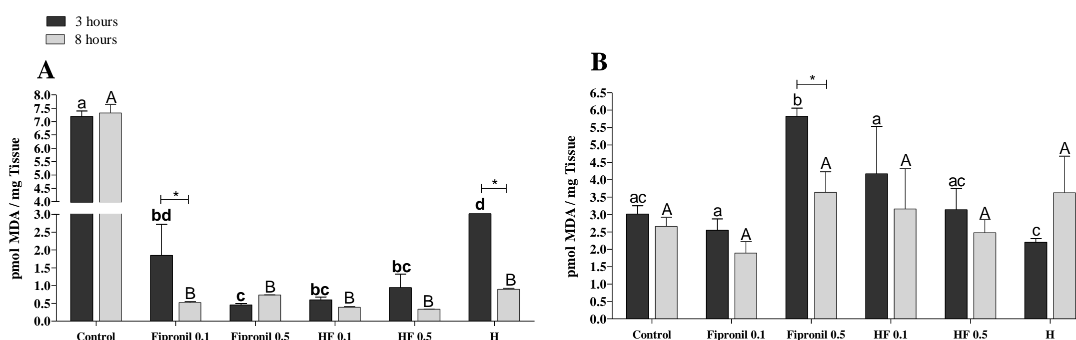

Fig. 3: Lipid peroxidation levels in **A:** gills e **B:** liver of *O. niloticus* exposure to fipronil (F 0.1 µg.L^{-1} e F 0.5 µg.L^{-1}), Hypoxia (H) and Hypoxia with Fipronil (HF 0.1 µg.L^{-1} e HF 0.5 µg.L^{-1}). Different lower letters indicate statistical difference between groups into the 3 hours period. Different capital letters indicate statistical difference between groups into the 8 hours period. Bars indicate statistical differenc between exposure times.

3.3 Genotoxic effects

The comet images showed that the tail lenght of the comet in the red blood cells of fish exposed to 0.5 µg.L^{-1} in combination with hypoxia increased significantly after 3 hours, compared to all other groups in the same period of exposure (Fig. 4A). On the other hand, the tail length of DNA from erythrocyte of fish exposed to hypoxia for 3 h was significantly lower compared to the other experimental groups. After 8 h, the lail lenght of DNA decreased significantly compared to the control group in the groups exposed to 0.1 and 0.5 µg.L^{-1} of fipronil, and in the group exposed only to hypoxia (Fig. 4A).

With respect to the percentage of DNA in tail, it was observed a significant increase in group exposed to 0.5 µg.L^{-1} of fipronil in combination with hypoxia for 3 hours, but a significant decrease in the groups exposed for 8 only to fipronil at 0.1 µg.L^{-1} and exposed only to hypoxia (Fig 4.B), in comparison to the respective control groups.

After 8 h, the group exposed to 0.5 µg.L^{-1} fipronil in combination with hypoxia presented higher percentage of DNA in tail than the groups exposed only to fipronil 0.1 µg.L^{-1} and the group exposed only to hypoxia.

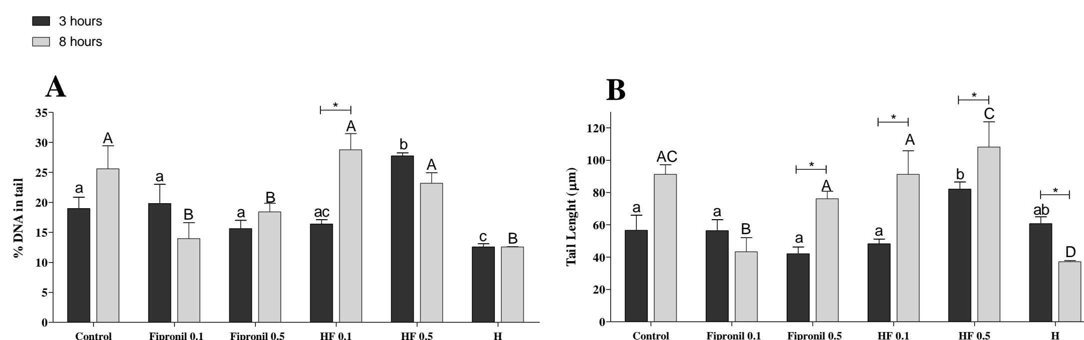

Fig. 4: DNA damage observed in the erythrocytes of *O. niloticus* exposure to fipronil (F 0.1 µg.L^{-1} e F 0.5 µg.L^{-1}), Hypoxia (H) and Hypoxia with Fipronil (HF 0.1 µg.L^{-1} e HF 0.5 µg.L^{-1}). **A:** shows the average of the tail length of the cells and **B:** shows percentage of DNA in tail of the cells. Different c f lower letters indicate statistical difference ($p<0.05$) between groups into the 3 hours period. Different capital letters indicate statistical difference ($p<0.05$) between groups into the 8 hours period. Bars indicate statistical differenc ($p<0.05$) between exposure times.

4. Discussion

In this study we observed that the exposure to fipronil (0.5 µg.L^{-1}) for 3 hours caused a significant decrease in the CAT activity in the gills compared to the other groups. Decline in enzyme activity due to pesticides has been documented (Sayeed et al., 2003; Classen et al., 2011; Freitas et al., 2017) and may have occurred due to a direct inhibition effect or due to an increase in superoxide radicals flow, which have already been reported to inhibit CAT activity and cause disturbances in the antioxidant system of fish (Monteiro et al., 2006; Classen et al., 2011). CAT inhibition was also observed in tadpoles (Boscolo et al., 2017; Freitas et al., 2017; Gripp et al., 2017) and rats (Kartheek and David, 2018) exposed to fipronil CAT decrease was also observed in the group exposed for 3 h to fipronil (0.5 ug.L^{-1}) in combination to hypoxia, confirming the inhibition effect of fipronil. Organisms exposed only to hypoxia did not present CAT alterations, suggesting that hypoxia did not affect this enzyme. Nevertheless, hypoxia seemed to increase susceptibility of fish gills to fipronil effects, since the fish exposed for 3 h only to fipronil at the lower concentration did not presented any alteration in CAT activity, while a significant decrease was observed at this fipronil concentration in combination to hypoxia. It is interesting to note that the short-term (3h) fipronil effects on CAT activity of gills were controlled after 8 hours,

since no differences in CAT activity were observed between experimental groups in this period, showing the ability of the animal to adjust their metabolism to the treatments over time.

In the liver, the inhibitory effect of fipronil was evident only after 8 hours of exposure, and only in those fish exposed to the lowest fipronil concentration, isolated or in combination with hypoxia. In contrary, CAT activity was increased in the liver of those fish exposed to hypoxia, independent on the presence or the absence of fipronil, after the 3 h hours of exposure. Due to the lack of statistical differences between the groups exposed to hypoxia and the groups exposed to hypoxia and fipronil, we believe that this result are more probably a response to hypoxia exposure, and possibly indicate a preparative antioxidant response to reoxygenation, as already proposed for numerous other hypoxia-tolerant animals (Almeida e Di Mascio, 2011; Hermes-Lima et al., 2015). Nevertheless, after 8 hours of hypoxia, CAT activity returned to values similar to the control group, possibly due to an inefficiency to maintain higher antioxidant levels after prolonged hypoxia, as also proposed (Oliveira et al., 2018). However, it is notable that the fish is able to maintain normal levels of CAT activity, even after 8 h of hypoxia, which elicit a great antioxidant adaptative response to hypoxic conditions. On the other hand, as previously mentioned, fipronil exposure was able to impair this capacity of CAT maintenance during hypoxia, demonstrating that fipronil does affect fish's mechanisms to deal hypoxic events.

The activity of GPx was increased in the gill after 3 h of hypoxia exposure, returning to basal levels after 8 h of hypoxia, suggesting a possible increase in ROS production, especially hydrogen peroxide and/or organic peroxides. A preparative mechanism for reoxygenation, as suggested for CAT increase, can be also suggested for the observed alteration of GPx activitye, since this enzyme was increased only in groups exposed to hypoxia. After 8 h of hypoxia, ROS production could decrease, leading GPx to return to basal levels, but in the presence of 0.5 $\mu g.L^{-1}$ of fipronil, ROS remained being produced, still stimulating GPx. In the liver, maybe due to its lower perfusion by oxygen compared to the gill, no such increase was observed in GPx activity, but the fish were able to maintain this enzyme unaltered, demonstrating the capacity of maintaining antioxidant defenses even at hypoxic conditions and fipronil exposure.

GST is an important phase II enzyme of xenobiotic biotransformation, and has also proposed to act on the excretion of acidic compounds accumulated during hypoxic periods (Di Ilio et al., 1996; Van der Oost, 2003). When compared to the control group, this enzyme was not altered in the gills, but it was increased after 3 h in the group subjected to hypoxia in comparison to the groups exposed to fipronil (0.1 and 0.5 $\mu g.L^{-1}$) in combination with hypoxia. This result suggests the involvement of GST on protection of the gill against metabolic products produced during hypoxia, but the presence of fipronil impaired this response. Interestingly, this pattern was maintained after 8 h: no differences in GST activity in comparison to the control group, but the group of fish submitted only to hypoxia presented higher GST activity in the gills compared to the groups submitted to hypoxia in the presence of both concentrations of fipronil. In the liver, fish exposed only to hypoxia for 3 h also presented higher GST activity compared to the control group, reinforcing the hypothesis of GST protection against acidic products generated during hypoxia. Moreover, it should be considered that some GST isoforms have also a peroxidase activity (Almeida et al., 2005; Singh et al., 2016), so the observed induction of GST in the gills and liver of fish submitted to hypoxia could also represent the instigation of antioxidant defenses during oxygen deprivation.

Among the hypoxia effects on GST activity, it was also noted that the activity of this enzyme in the gills of fish exposed to 0.5 $\mu g.L^{-1}$ of fipronil was higher than the GST activity observed in the groups exposed to hypoxia and fipronil (both concentrations), and compared to the group exposed to 0.5 $\mu g.L^{-1}$ for 3 h. GST activity was also increased after 8 h of exposure to fipronil at 0.1 $\mu g.L^{-1}$ in combination to hypoxia compared to the same group at 3 h. In the liver,fish exposed to fipronil at 0.5 $\mu g.L^{-1}$ in combination with hypoxia also presented higher GST activity compared to all the other experimental groups. These increases could indicate the participation of GST in the biotransformation of fipronil, as already proposed in mammals (Han et al., 2016).

Although fipronil and hypoxia stimulated alterations in the activities of GPx and GST in the gill, they were not reflected in GR activity, since no alterations were observed in this enzyme in the gills between different experimental groups. It can be supposed that the maintenance of basal activity of GR by the fish was sufficient to maintain recycling GSH for GST and GPx in the gill. In the liver, the increases in GR

activity after 3 h of hypoxia in combination with 0.1 µg.L^{-1} of fipronil, and after 8 h of fipronil exposure to 0.1 µg.L^{-1} or exposure only to hypoxia indicates an effort of this organ to maintain GSH at adequate levels, as a response to its consumption due to increased ROS production or GST activity.

The alterations in antioxidant enzymes in the liver but especially in the gills, could suggest the generation of ROS due to fipronil exposure and hypoxia period, isolated or in combination. However, only the fish exposed for 3 h to the higher concentration of fipronil presented significant increases in lipid peroxidation levels in the liver, compared to the control group, which could be related to increased ROS production and/or CAT inhibition. The other experimental groups did not presented any alteration in MDA levels in comparison to the control group, indicating that modulation in antioxidant defenses due to fipronil and/or hypoxia were sufficient enough to avoid lipid peroxidation, after 3 and 8 h. In contrast, MDA levels in the liver of fish from all experimental groups were significantly lower than controls, after both 3 and 8 h. This result suggest the instigation of massive antioxidant protection in treated groups, causing excessive ROS elimination and resulting in MDA levels below the control levels. Numerous studies have also reported decreased lipid peroxidation levels in organisms submitted to environmental stress such as environmental hypoxia (Murata et al., 1994; Turkan et al., 2005; Garcia et al., 2015) and contaminant exposure (Felicio et al., 2015; Freitas et al., 2017), an effect that was suggested to be a reflection of antioxidant induction. However, in the liver only discrete positive modulation were observed on the measured antioxidant enzymes. It can be possible that other antioxidants that were not evaluated in this study, such as superoxide dismutase, peroxyredoxins, GSH levels and lipophilic antioxidants (i.e. α-tocopherol, ascorbic acid and carotens), could be also increased in the experimental groups in comparison to the control group, contributing to decrease MDA levels. Another hypothesis is that the activity of aldehyde dehydrogenase (ALDH) was induced, resulting in the metabolization of MDA excess generated due to oxidative stress. In a previous study (Garcia, 2016), we showed that diesel oil induced ALDH activity with a concomitant decrease in MDA levels in the liver of *Astyanax sp*. However, these hypotheses remains to be further confirmed.

With respect to DNA damage in erythrocyte, the results showed that the combination of hypoxia with fipronil at 0.5 µg.L^{-1} for 3 h caused a significant increase

in the amount of DNA in tail and in tail length, in comparison to the control group, indicating a deleterious situation for the fish in this experimental group, increasing DNA damage. In contrary, fish exposed for 8 h only to fipronil (both concentrations) or only to hypoxia, presented lower amount of DNA in tail, while fish exposed for 8 h to fipronil at 0.1 $\mu g.L^{-1}$ or submitted to hypoxia showed decreased tail length in comparison to the control group, suggesting the stimulation of DNA repair mechanisms.

In summary, our results demonstrated that fipronil exposure or hypoxia insult are deleterious conditions for fish, causing significant alterations in antioxidant enzymes and damage levels to biomolecules. More relevant, we noted that fipronil have an inhibitory effect on CAT activity while hypoxia seems to stimulate antioxidant enzymes, effects that were more evident in the gill, compared to the liver. Moreover, most alterations occurring after 3 h were normalized after 8 h, demonstrating biochemical adjustments of fish organs to fipronil and/or hypoxia exposure. In addition, the significant decrease seen in MDA levels in the liver suggest a positive modulation of antioxidant defenses, an effect that was not noted in the measured antioxidant enzymes. Alternatively, this reduced MDA levels would be a consequence of its increased metabolism, i.e. by an induction of ALDH, an hypothesis that remains to be clarified. Finally, the combined exposure to fipronil and hypoxia to increase the susceptibility of the fish to negative physiological effects, especially considering the higher DNA damage in erythrocytes of fish exposed to the mixed exposure. Data obtained in this study are of great relevance considering that fish can be exposed to chemical contaminants in aquatic environments with low dissolved oxygen, possibly affecting the adaptative mechanisms developed by fish to deal with hypoxic environments.

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CAPÍTULO III

Combined effects of the insecticide Fipronil and temperature on the metabolism of the benthic fish *Dicentrarchus labrax*.

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Abstract

A comprehensive study on the effects of food administration of Fipronil (80% active ingredient of REGENT® 800WG) under two temperatures was carried out in juvenile sea bass *Dicentrarchus labrax*. The temperature was that natural during the winter period and another mimicking a 3ºC increase, as forecasted under a realistic climate change scenario. The parameters were measured in the fish acclimated to the two temperatures for 2 weeks (t=0) and after further 7 and 14 days fed with spiked food (10 mg/kg fish) and a depuration period of another 7 days (t=21). Metabolic and oxidative stress markers were assessed in different tissues: Acetylcholinesterase (AChE), propionylcholinesterase (PrChE) and Butyrylcholinesterase (BuChE) were analysed in muscle S10 fraction; lactate dehydrogenase (LDH) was analysed in a portion of liver (S9); Glutathione S-transferase (GST) activity was measured of the liver; Catalase (CAT), total glutathione peroxidase (GPX) and glutathione reductase (GR) were determined in the liver cytosol; Estradiol (E_2), Testosterone (T) and 11-Ketotestosterone (11-KT) were analyzed in the plasm. The results of this study with *D. labrax* fish revealed that fipronil administered through the food, under the influence of different temperatures, impaired the metabolic and antioxidant responses of exposed animals, as well changes in endocrine signaling. These data are of great relevance to understand the effects of environmental variations due to climate change and the influence of the presence of chemical compounds in natural environments.

Palavras-Chave: fipronil, oxidative stress, endocrine disrupter. enzymatic markers

1. Introduction

Human activities have been considered the main causes of climate change (CC) observed in the world (Moron et al. 2006). There is currently consensus that in the coming decades there will be notable increases in temperature, acidification and salinity of marine water bodies around the world (IPCC, 2014). The effects caused by the CC have generated great concern to the scientific community, which sought to assess the consequences of these changes for wildlife and humans. For example, it was observed that the temperature increases cause impact on the oceans acidification induced by CO_2, modifying the thermal tolerance of marine ectotherms (Pörtner and Farrell, 2008; Lannig et al., 2010). In this sense, a similar case of the Mediterranean Sea was also analyzed (Calvo et al., 2011). However, the indirect effects caused by CC in benthic species, for instance, is still little known, mainly in relation to the interaction potential of these changes with foreign chemicals (Hooper et al., 2013). The change of physical factors in conjunction with chemical exposures can act synergistically and increase the consequences of exposures to marine organisms, including fish and bivalves, because it can significantly change the disposal and action of these chemicals in the environment (Sokolova and Lannig, 2008; Carregosa et al., 2014; Freitas et al., 2015).

Coastal marine species are constantly subject to seasonal and periodic changes in the physico-chemical parameters of water and develop different sets of physiological, behavioral and morphological compensations to deal with these natural environmental fluctuations, which have important consequences on population dynamics and ecological processes (Ganugapenta et al., 2015). Climate changes and the presence of toxicants in the aquatic environment may interfere in the normal adaptation of animals to environment constants, mainly when facing acidified environments and higher temperatures, due to the increased rates of chemical and biological reactions (Middlebrooks et al., 1973). For the European Sea bass, temperature is a basic abiotic factor that regulates its physiology and metabolism, since that is a coastal and estuarine species that spends most of its life cycle in these environments under severe changes of temperature and, as a coastal benthic species, is exposed to many substances derived from wastewaters that finally reach the sea and can be stored in the sediment (Costas et al., 2012; Gaw et al., 2014; Blair et al., 2015; González-Mira et al., 2016). In addition, the *D. labrax* species is of great economic importance because of its wide use for human

consumption, which can be affected by the temperature rise because of climate changes (Almeida et al., 2015).

Among the sources of entrance and dispersion of toxic substances in marine and coastal environments, agricultural activities stand out. In the Mediterranean, the main economic interest in the Ebro Delta region, where the fish *D. labrax* coexists with other related species, is agricultural practices, aquaculture production and fishing activities (Sànchez-Nogué et al., 2013). Many pesticides are used in these regions mostly during spring and these chemicals reach the surrounding bays and marine waters at the end of the spring-summer period (Köck-Schulmeyer et al., 2010). This coincides with a period of great species diversity that can affect non-target species. Thus, in this scenario, it is believed that the benthic species that inhabit or are cultivate in these bays are increasingly threatened by the effects of exposure to local pollutants associated with agricultural practices and by modifications in water physical parameters caused for climate changes.

The phenylpyrazole Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-(trifluoromethylsulfonyl) pyrazole-3-carbonitrile), classified as highly toxic (Class II) is one of the insecticides most used in controlling a wide variety of insects harmful to crops worldwide, including those resistant to pyrethroid, organophosphates and carbamate insecticides (Stefani Margarido et al., 2013). In Europe, it is mainly used in crops of maize, rice and in the treatment of seeds for the planting of sunflower. Its use was banned by the European Union in April/2013 (Directive ECC Nº 781/2013), indicating that it is very harmful to non-target species. Spain, the largest Fipronil consumer in Europe, did not adhere to the directive because of the few on-site studies that proved their toxicity. However, there is strong evidence that soils, aquatic environments and plants in agricultural environments or their neighboring areas are contaminated with several of these and other related contaminants (US Environmental Protection Agency, 1996; Bonmatin et al., 2015).

Some studies have demonstrated the potential action of Fipronil as an endocrine disrupter in various organisms such as fish, crustaceans, mammals (Volz and Chandler, 2004; Ankley et al., 2009; Leghait et al., 2010; Beggel et al., 2012; Bencic et al., 2013; Lu et al., 2015). Fipronil and other insecticides may interfere in the endocrine system of animals and cause disruption in the production and action of natural hormones, because they bind to the estrogen receptor and alter the reproductive function in exposed

organisms (Mnif et al., 2011). Exposure of freshwater fish larvae (*Pimephales promelas*) for a short time to the insecticide Fipronil, caused vitellogenin production up-regulation and delayed development of germ cells in male larvae, indicating that Fipronil probably affects the endocrine system, in particular the estrogenic, and the gonadal development of these fish (Beggel et al., 2012). Alterations in the endocrine system of fish by environmental estrogens, as Fipronil, has caused great concern, mainly for its mechanisms of action in the reproductive function of the organism, since changes in the endocrine signaling lead to reproductive failure. Thus, the action of Fipronil as an endocrine disrupter can be applied as a parameter to evaluate the physiological signals, such as growth and development of the organism (Filby et al., 2006).

In natural systems, the action of pesticides can be influenced by temperature, which is a critical variable for the development of benthic species for example and influence the final degradation of the chemical compound (Lyons et al., 2011; Hiebenthal et al., 2013; Freitas et al., 2015). Changes in biotransformation mechanisms of xenobiotics may promote significant impacts on the control of many bodily functions, making the animals more vulnerable. It has been reported that several isoenzymes of the P450 family are involved in the biotransformation metabolism of Fipronil and in comparison, to other contaminants it has been demonstrated to be the most potent and toxic inducer of CYPs isoforms (Wang et al., 2016). Exposure to chemical compounds that induce the biotransformation in conjunction with environmental factors is a concern for benthic fish, such as *D. labrax*, since its metabolism can be regulated by the temperature of water (Almeida et al., 2015). Studies have also shown that increases in water temperature can modulate metabolism in D. labrax by altering the activity of antioxidant enzymes and biotransformation, especially when in conjunction with chemical exposures (Almeida et al., 2015; Maulvaulta et al., 2017). In addition, changes in temperature of water can also generate changes at the cellular and molecular levels that can be evaluated and measured by biochemical responses.

Thus, considering the warming of the sea driven by anthropic actions, such as increasing concentrations of greenhouse gases that bring threats to coastal ecosystems, the aim of the present study is to identify the sensitivity of the species *D. labrax* in

terms of energy needs, as consequence of changes in temperature, including the additional effect of another stress factor, such as exposure to the insecticide Fipronil.

2. Material and Method

2.1 Design Experimental

D. labrax juveniles that were used in this experimental were provided by IRTA-Generalitat de Catalunya (Sant Carles de la Rápita, Spain). Specimens were chosen between 75-100 g and transported and maintain in the ZAE facilities of the ICM-CSIC. The animals were acclimated for 2 weeks prior to the experiment in 2400L round fiberglass tanks (180 mm x 85 cm) using sea filtered water (sterilized Sand filter 50 microns) under natural conditions of temperature (range 11 – 13 ºC). The water flow was renewed 24 times a day. Before the experimental conditions, the fish were randomly assigned to 4 fiberglass tanks of 680L (100 mm x 90 cm) (~19 individuals per tank) with the same conditions as above for the start to experimental exposure. For treatments to temperature, eight organisms *D. labrax* were maintained under ambient conditions of temperature at the rearing conditions of the aquaculture facilities (e.g.T: 13ºC; pH: 7,0), and other eight organisms were submitted to treatments (T: 16ºC) for two weeks (T0).The desired parameters were established gradually by lowering the temperature at a rate of about 1°C/day. Physical water conditions observed were T: 13.35 and 16.55 ºC; O_2: 6.63 mg.L; pH: 7.68; salinity: 37.80. During this period, the fish were feed daily with pellets dry food (L-4 Optibass 2P, Skretting España). After the temperature exposure (14 days), fish were submitted to the treatment with the same ration commercial contaminated with Regent®800WG (80% Fipronil) at a concentration of 10mg MT Kg^{-1}, with a daily average intake of 15 gr of pellet feed per condition. The experimental diet was prepared following the method of (Blázquez et al., 1995; Konwick et al., 2006). The pellets dry food was sprayed with the commercial formulation of fipronil dissolved in ethanol. The ethanol was allowed to completely evaporate at room temperature before experimental diet. After 14 days exposure to fipronil treatment, the fish were subjected to depuration period in the different temperatures for another 7 days, feed with pellets no contaminated with fipronil. The

fish were sampled in T0, 7, 14 and 21 days of exposure and for each treatment eight fish were collected.

2.2 Analysis and measured parameters

Fish Sampling

After experimental period, biometrics were recorded, such as total length and weight and about 1 mL of blood from the caudal vein was withdrawn using heparinized syringes. Plasma was obtained by centrifugation at 5.000 g x 5 minutes at 4 ºC. Fish were killed by spinal cord severing, and then dissected and collected the liver, gills and muscle and frozen in liquid nitrogen. Handling of the fish was done according to national and institutional regulations of the Spanish Council for Scientific Research (CSIC) and the European Directive 2010/63/EU.

Tissue Preparation

A portion of muscle were homogenized using ice-cold buffer phosphate (50 mM pH 7,4) in a 1:5 (w:v) ratio using a polytron® homogeniser. The homogenates obtained were centrifuged at 10.000 *g* x 30 min. The supernatant was aliquoted and stored at -80ºC for further biochemical determinations. For microsomes and cytosol fractions, the livers were homogenized in ice-cold buffer phosphate (100 mM pH 7.4) containing150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1 mg/mL trypsin inhibitor and 1 mM ethylenediaminetetraacetic acid (EDTA) in a 1:4 (w:v) ratio using a Polytron® blender. The obtained homogenate was centrifuged again at 100 000 g × 60 min at 4 °C. The microsomal pellet obtained was dissolved in the above homogenization buffer, which also contained 20 % glycerol in a 2:1 (w:v) ratio (Koenig et al., 2013).

Biochemical Analyses

All reactions were carried out in triplicate at 25ºC or using the kinetic mode (Magellan v6.0) on a TECAN Infinite 200 microplate reader. All protocols have been described in detailed elsewhere (Solé et al. 2014).

Esterases

AChE, PrChE and BuChE were analysed in muscle S10 fraction following Ellman (1961) protocol using a 1 mM substrate concentration in all cases, at 405 nm. CbE was measured in the factions of the liver (S9) using as substrates either 250 µM αnaphtyl acetate (αNA), αnaphtyl butyrate (αNB), βnaphtyl acetate (βNA) at 235 nm (Mastropaolo and Yourno 1981) and 1 mM ρ-nitrophenyl acetate (pNPA) and ρ-nitrophenyl butyrate (pNPB) at 405 nm (Hosokawa and Satoh 2002).

Energy Metabolism Enzymes

LDH (lactate dehydrogenase) was analysed in a portion of liver (S9) following adaptation of the Vassault et al. (1983) using NADH (200 µM) and pyruvate (1 mM) as final well concentrations at 340 nm.

Conjugation enzyme activities

Glutathione S- transferase activity (GST)

Glutathione S-transferase (GST) activity was measured using 25 µl of the cytosolic fraction of liver using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione (GSH). The activity rate was measured for 5 min at 340 nm (Habig et al. 1974) and expressed as nanomole per minute per milligram protein.

Antioxidant enzymes and LPO levels

Catalase (CAT), total glutathione peroxidase (GPX) and glutathione reductase (GR) were determined in the liver cytosol. CAT activity was measured as a decrease in absorbance at 240 nm using H_2O_2 (50 mM) as substrate, GPX and GR at 340 nM used cumene hydroperoxide (CHP; 0.625 mM) and oxidized glutathione (GSSG; 0.9 mM) as respective substrates and NADPH as cofactor in both assays at 340 nm. LPO was measured in the muscle fraction using a colorimetric method using 1methyl-2-phenylindole and the quantification, in respect to the standard 1,1,3,3-tetramethoxypropane, was made at 586 nm.

Protein determination

Total protein content of the samples was determined by the Bradford (1976) method adapted to microplate, using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.1-1 mg/ml) as standard. The absorbance was read at 595 nm.

Hormones Measurement

Sex steroid analysis

Plasma levels of hormones were measured by a specific ELISA method, following the protocol previously described (Guzman et al., 2008). The plasma samples with undetectable levels were recorded considering the detection limit. Plasma samples were analyzed at a minimum 10-fold dilution, to avoid potential interferences of plasma metabolites in the assay. Plasma levels of all three steroids (Estradiol (E_2)), Testosterone (T) and 11-Ketotestosterone (11-KT) were analyzed by their respective specific ELISA method.

3. Statistical Analysis

The presence of outliers was evaluated, and the normality of the data was verified using the Shapiro-Wilk test. For the parametric data, the statistical differences were identified using the ANOVA test, followed by a *post hoc*Tukey test. Data that do not follow the normality and homoscedasticity assumptions were submitted to the Kruskal-Wallis non-parametric test, followed a Student's t-test. Values of $p<0.05$ was considered as a reference to assign statistical significance.

4. Results

Biological Parameters

In the Table 1 biometric characteristics of the fish and the water quality in each treatments and temperature are indicated.

Table 1. Biometric characteristics (weight, length) of *Dicentrarchus labrax* exposed to different temperatures and food administration with fipronil insecticide, measured at the exposure period, and means values of parameters of water quality (O2, temperature, pH, salinity) measured during the exposure time.

Temperature	Treatment	Parameters						
		Fish Weight (g)	Fish Lenght (cm)	O_2	Temperature (ºC)	Salinity	pH	Food (g)
13ºC	Control	125.07 ± 52.18	20.50 ± 2.67	6.62 ± 0.26	13.45 ± 0.24	37.65 ± 0.07	7.83 ± 0.26	14.28 ± 5.53
	F7	174.27 ± 17.65	23.03 ± 0.59	6.44 ± 0.16	13.37 ± 0.17	37.85 ± 0.07	7.14 ± 0.04	11.37 ± 2.34
	F14	145.80 ± 27.46	21.65 ± 1.62	6.94 ± 0.01	13.38 ± 0.09	37.90 ± 0.01	7.95 ± 0.07	8.95 ± 2.15
	Depuration	164.99 ± 9.11	22.56 ± 0.82	6.70 ± 0.33	13.13 ± 0.11	37.85 ± 0.07	7.89 ± 0.21	8.29 ± 1.67
16ºC	Control	138.14 ± 40.01	20.68 ± 2.14	6.44 ± 0.80	16.95 ± 1.01	37.62 ± 0.10	7.67 ± 0.46	13.52 ± 4.15
	F7	139.76 ± 43.53	21.56 ± 2.30	6.73 ± 0.10	16.31 ± 0.14	38.15 ± 0.07	7.59 ± 0.09	13.13 ± 1.63
	F14	153.21 ± 52.72	21.50 ± 2.49	6.83 ± 0.19	16.30 ± 0.09	38.10 ± 0.01	7.55 ± 0.49	13.26 ± 3.23
	Depuration	163.81 ± 33.24	22.62 ± 1.82	6.54 ± 0.50	16.36 ± 0.10	38.15 ± 0.07	7.70 ± 0.38	10.17 ± 1.64

* Data are expressed as means and standard derivation.

Esterases and LPO levels

Cholinesterases in muscle (AChE, PrChE, BuChE) did not present significant differences for any of the treatments analyzed (Table 2). LPO levels in the muscle were increased in all groups compared to control in the two temperatures tested (13 and 16ºC). In the groups exposed to temperature of 16ºC, it was observed significant differences between the treatments. The highest levels of LPO were found in the groups exposed to the depuration period.

Different substrates of Cbe (αNA, αNB, βNA, pNPA, pNPB) were analyzed in the S9 portion of fish liver, however, a significant difference was observed between the groups exposed to the treatments only for substrates αNB to pNPA (Figure 1). It was observed that in the groups exposed to the temperature of 13ºC all groups presented values significantly higher than the control for the substrate αNB. In the temperature of 16ºC, the group exposed to treatment with fipronil for 14 days presented values significantly higher than the control and the exposed group for 7 days. No difference was observed between the two temperatures for any of the treatments.

For the pNPA substrate, we observed that at both temperatures the groups exposed to the depuration period presented significantly lower values when compared to the group exposed to treatment with fipronil for 14 days, and in the groups exposed to

the temperature of 13ºC, also it was observed a significant difference when compared to the group exposed to treatment with fipronil for 7 days (Figure 1).

Oxidative stress response

CAT activity in the groups exposed to the temperature of 13ºC was significantly higher in the cytosolic fraction of the liver of the groups exposed only to the temperature, to the feed contaminated with fipronil for 7 and 14 days, compared to the group exposed to depuration period, after treatment for 14 days with food contaminated with fipronil. Alterations in GPx and GR activity were not observed. With respect to GST, it was observed higher activity in the groups exposed to temperature of 16ºC in the depuration period, compared to the groups exposed to temperature control and 7 days to contamination with fipronil (Table 2).

Metabolic enzymes

LDH activity (in nmol/min/mg prot) did not present significant differences for any of the treatments analyzed (Figure 1).

Sex steroid in Plasma

Steroids were analyzed in the plasma of individuals exposed to temperature only (13 and 16ºC), fipronil for 7, 14 days and depuration period in each temperature. No significant differences in plasma estradiol levels were observed between the exposed and control groups at the temperature of 13ºC. However, in the temperature of 16ºC all the groups presented higher levels of E_2 compared to control, besides that in the groups exposed to 14 days and in the depuration period, it was observed that at 16ºC E_2 levels were higher in relation to those groups in the temperature of 13ºC (Figure 2).

The T levels in plasma at 13 ºC were lower for all groups compared to control (Figure 2). At 16 ºC the groups exposed to fipronil for 14 days in the food showed increased levels of testosterone compared to control and was significantly higher than the same group at the temperature of 13 ºC. It was not observed significant differences for any of the treatments analyzed for the 11-KT hormone (Figure 2).

Table 2. Enzymatic activities of Catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) levels in the cytosolic fraction of liver and malondialdehyde (MDA) and Cholinesterases (AChE, PrChE, BuChE) in the muscle exposed to different temperatures and feed contaminated with fipronil.

Temperature	Treatment	Biochemical Biomarkers							
		CAT*^	GPx**^	GR*^	GST*^	MDA*^{B}	AChE*^	PrChE*^	BuChE*^
13ºC	**Control**	103.6 ± 35.2 a	19.09 ± 33.51	7.48 ± 2.87	36.93 ± 4.89	6.70 ± 3.86 a	18.24 ± 5.78	11.18 ± 2.79	13.07 ± 3.90
	F7	90.7 ± 24.5 ab	6.60 ± 1.13	6.03 ± 2.43	39.49 ± 4.26	10.69 ± 2.72 b	15.44 ± 4.13	9.92 ± 3.36	11.27 ± 4.21
	F14	92.8 ± 32.1 a	6.70 ± 0.92	6.84 ± 1.92	42.83 ± 9.95	10.48 ± 2.24 b	18.19 ± 5.11	10.75 ± 2.76	12.77 ± 4.08
	Depuration	63.8 ± 29.0 b	7.16 ± 1.53	7.98 ± 2.38	43.25 ± 7.67	11.79 ± 3.17 b	16.92 ± 5.06	10.48 ± 2.95	10.35 ± 3.64
16ºC	**Control**	96.7 ± 20.3 ab	8.11 ± 1.13	7.39 ± 1.79	39.44 ± 7.41 a	5.04 ± 2.16 a	17.05 ± 4.79	10.02 ± 2.37	12.43 ± 2.53
	F7	108.9 ± 24.7 a	6.63 ± 1.06	7.19 ± 2.64	36.44 ± 6.87 ab	8.15 ± 3.06 b	15.34 ± 2.84	8.99 ± 1.89	10.25 ± 2.18
	F14	111.1 ± 17.7 a	7.12 ± 1.47	5.55 ± 2.97	48.27 ± 7.13 ac	9.26 ± 3.14 c	17.59 ± 6.18	10.75 ± 3.12	13.16 ± 4.35
	Depuration	76.8 ± 32.0 b	7.96 ± 0.86	7.84 ± 3.31	51.04 ± 16.39 c	9.80 ± 2.21 d	17.30 ± 4.39	9.96 ± 3.15	11.15 ± 4.61

Different Letters - Statistical difference compared to the groups into each temperature ($p<0.05$).
* Means and standard derivation (parametric data) ** Mean and standard derivation (non-parametric data).
^ U.mg protein^{-1}; $^{\mathbf{B}}$ nmol/g tissue.

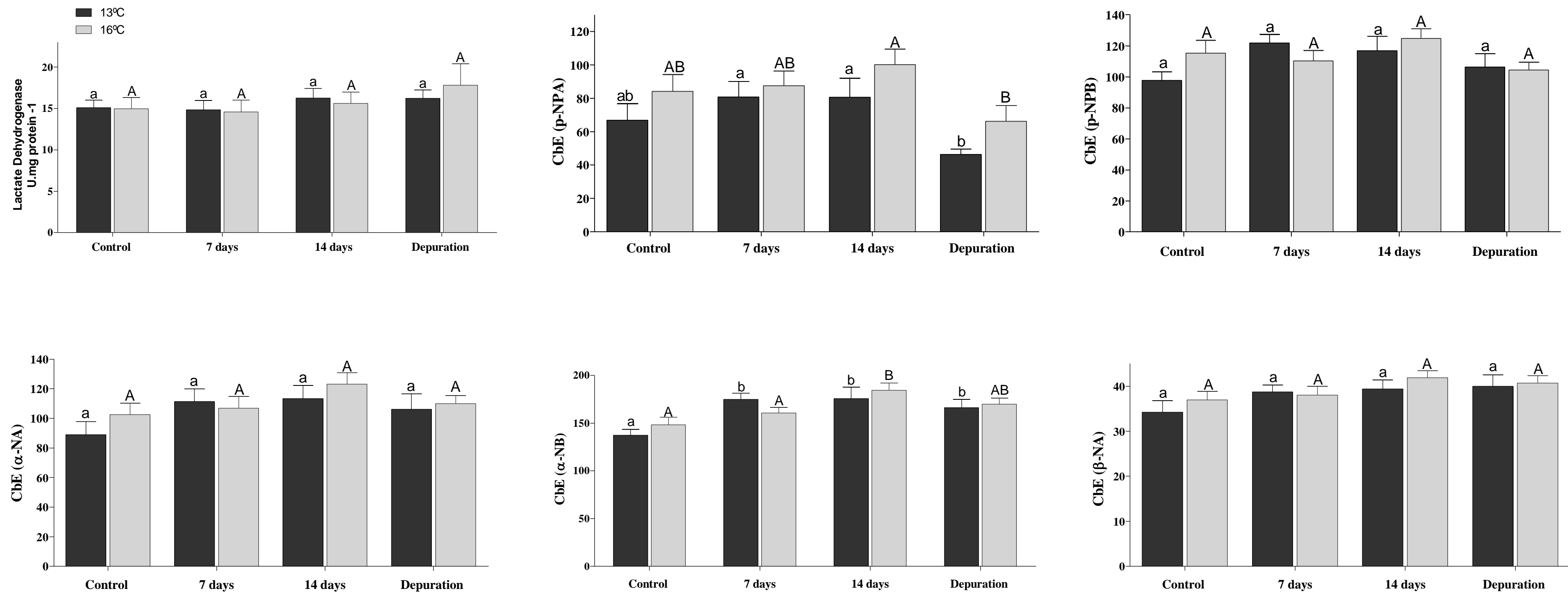

Figure 1. Lactate dehydrogenase (LDH) and type B esterase activities (in nmol/min/mg prot) for S9 of liver portion of *D. labrax* juvenils, expressed as means and standard derivation. * Different Letters - Statistical difference compared to the groups into each temperature (p<0.05).

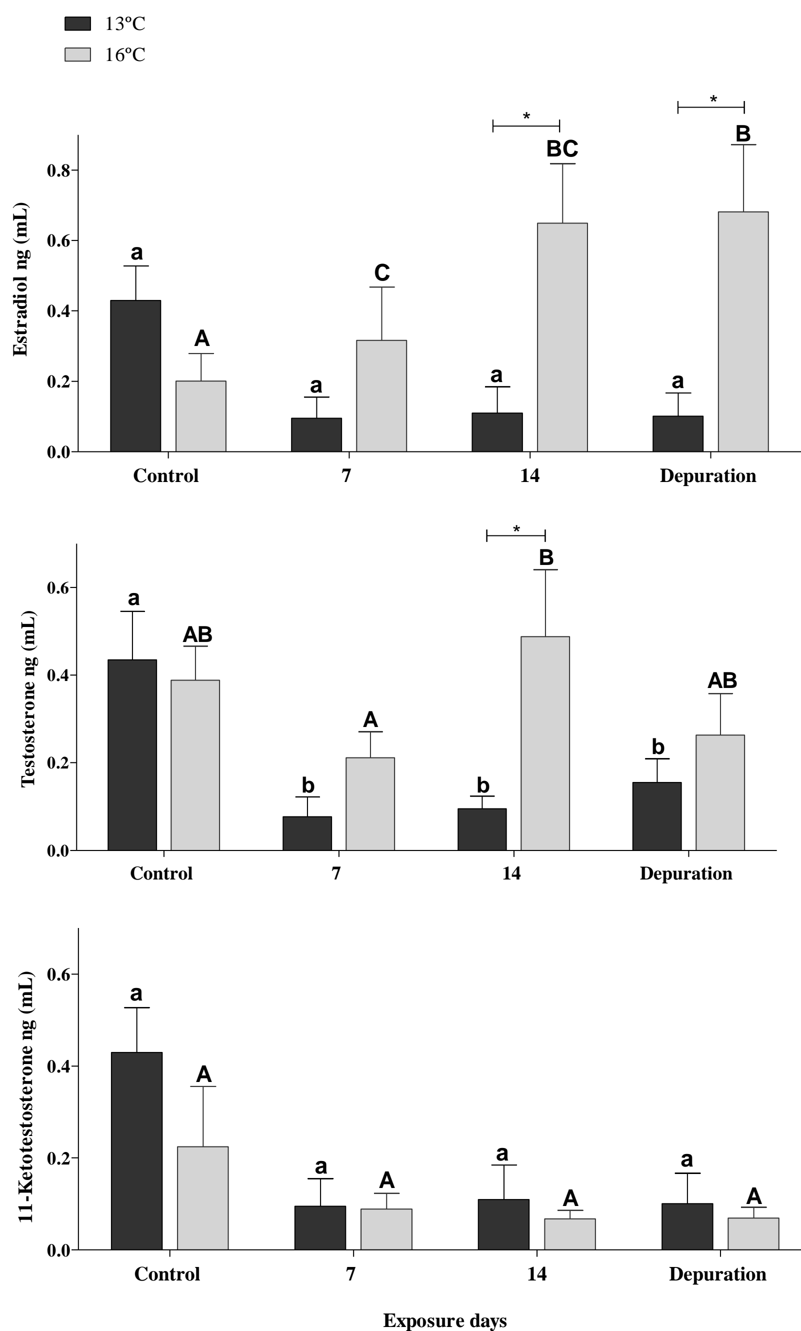

Figure 2. Steroids hormones in plasm of Sea bass (*D. labrax*) after exposure to pellet feed contaminated with 10 mg/g of fipronil. Values are mean ± SEM ($p < 0.05$). Distinct small letters indicate significant differences between the groups exposed to 13 ºC temperature, and capital letters indicate significant differences between the groups exposed to 16ºC temperature. Bars indicate significant differences between the temperatures.

4. Discussion

The results obtained in this study demonstrated that the exposure to fipronil under different temperatures induced changes in the metabolism as well as in the hormonal levels of the European Sea bass fish. Effects were observed on the activity of the esterases in the S9 fraction of the liver of the fish. Among the analyzed substrates, αNB and pNPA were the most responsive among the treatment groups. Exposure to fipronil for 7 and 14 days at 13 ° C induced CbE activity measured with αNB substrate compared to control, a result also observed in the group exposed to the depuration period. At 16ºC CbE induction was observed only in the exposed group for 14 days compared to the exposed group for 7 days and the control, which was also observed for the substrate pNPA. This result may be related to the presence of fipronil, since these substrates present greater selectivity to the presence of pesticide (Solé et al. 2012). In addition, some studies have indicated that CbE can be modulated by a wide range of chemicals, as it participates in metabolism and detoxification reactions (Ribalta et al. 2015, Solé et al. 2014, Solé e Sanchez-Hernandez 2015).

There were no significant differences in the groups between the temperatures for this marker, which reforms the idea that the presence of fipronil has induced cholinergic activity, since these enzymes catalyz efficiently the hydrolysis of many products e and can be said to be involved in the process of detoxification or metabolic activation of various chemical contaminants (Hosokawa, 2008). Besides that, we observed that in groups exposed to the clearance period at both temperatures tested there was CbE inhibition (pNPA) compared to the other treatments e this effect may be from due the metabolization of fipronil previously, considering that the performance of certain compounds can occur slowly and form stable conjugates, resulting in the abnormal functioning of these enzymes and later accumulation of acetylcholine in the synaptic cleft. This accumulation generates excessive stimulation at the muscarinic and nicotinic receptors of CbE, which can promote an acute cholinergic syndrome and trigger long-term toxicity (Silva, 2015).

It is also known that most of the studies involving oxidative stress are related not only to CbE activity, but also to antioxidant enzymes and biotransformation, such as SOD, GPx, CAT and GST. In this study, we observed significant increases in CAT activity in the cytosolic portion of the liver in the groups exposed to fipronil at 13ºC and 16ºC compared to the group exposed to the period of depuration. CAT is an

enzyme that is involved in the detoxification of reactive oxygen species by the hydrolysis of H_2O_2 and can be modulated by environmental pollutants (Barreiros et al., 2006) Thus, CAT activity increased or close to control evidences the ability of Fipronil to induce oxidative stress by increasing reactive oxygen species, which activates the antioxidant mechanisms of organisms to protect themselves from the damages caused by the excessive production of free radicals. These results agree with previous results that observed an increase in CAT activity in fish (Lushchak and Bagnyukova, 2006; Almeida et al., 2015). However, a decrease in CAT activity in the group exposed to the clearance period for both temperatures was observed, in addition to an increase in GST activity in the clearance period compared to the group exposed to 7 days to fipronil at 16ºC. This change in enzyme behavior after treatment with fipronil shows that the time and presence of the chemical contaminant influences the response of the enzyme and that the induction of the CAT enzyme during periods of exposure to fipronil was not sufficient to overcome the oxidative stress, considering that concomitant to this also was observed a significant increase in the levels of lipid peroxidation in the fish muscle exposed to all the treatment compared to the control in the two temperatures.

The significant increase observed in the activity of the GST enzyme in the group exposed to the clearance period suggests that changes in the absorption, distribution and biotransformation of the compound may have occurred after the exposure period, que influencing the normal functioning of the enzyme, since it is involved in phase II reactions of biotransformation of xenobiotics (Singh et al., 2016). Sea bass kept on treatment with fipronil under different temperatures did not show significant variations in the activity of the glycolytic enzyme LDH in the S9 portion of the liver for none of the treatments. LDH is one of the representatives of the aerobic and anaerobic pathways for energy production and catalyzes one of the major stages of the citric acid cycle, antioxidant defenses, being crucial for the regeneration of NADPH in glutathione conjugation pathways (Lee et al. al. 2002) and suggests that energy metabolism was not compromised during treatment.

Changes in endocrine signaling may affect important parameters in the development of organisms, especially in the reproduction (Filby et al., 2006). In many species, 17β-estradiol (E_2), Testosterone (T) and 11-ketotestosterone (11-KT) steroid hormones are abundantly produced in gonadal tissues under the control of pituitary

gonadotrophins (GTH) and are essential for critical gametogenesis (Wallace and Browder 1985; Taghizadeh et al., 2013). Increase plasma estradiol (E_2) levels were observed in this study in all treatment groups compared to the control at 16 °C. Many pesticides can interfere with hormonal function and thus may cause negative effects on the reproductive system (Almeida et al., 2018. Increases in estradiol levels observed in the groups exposed to fipronil as well as in the depuration period exemplify the negative effect of pesticides on hormonal function, since the contaminant probably interfered in the recognition and binding of estrogen receptors, because when an endocrine disruptor or its metabolites binds and does not activate the receptor, acting as an antagonist, it is suggested that there are increases in estradiol concentration and no inhibition (Schettler et al., 2006; Bretveld et al., 2006).

The effect of fipronil as an endocrine disruptor has also been observed in other studies with larval fathead minnow (Beggel et al., 2012), female copepods (Volz and Chandler, 2004), in vitro (Lu et al., 2015) an in fish (Ankley et al., 2009). In addition, it has already been documented that increases in estradiol levels may have a stimulatory effect on aromatase activity in Asian sea bass, which further stimulates E2 production and ovarian development, and ultimately can cause sex inversion in female (Guiguen et al., 2010). Temperature is also a factor that influences this sexual differentiation and the specification and morphology of the gonads (Matsumoto e Crews, 2012). The groups exposed to fipronil under the temperature of 16ºC presented significant increases when compared to the same group under the temperature of 13ºC and this result can be e esse this result may be related to the ability of estradiol to stimulate aromatase, which may lead to a considerable increase in females, as observed in Catfish (Patino et al., 1996) and *Dicentrarchus labrax* (Blazquez et al., 2008). The present study also clearly showed an increase in testosterone at the highest temperature in the group exposed to fipronil for 14 days in comparison to the same group at lower temperature, reinforcing the idea that in high temperatures can influence in the plasma concentrations of sex steroids. In the temperature of 13 ºC, the level of testosterone (T) decreased in all treatments compared to the control. Similar results were observed in Asian sea bass fish (*Lates calcarifer*) exposed to different temperatures (Athauda et al., 2012). This result suggests that this decrease is related to an action of the contaminant as an endocrine disruptor able of inhibiting the enzymes involved in steroid oogenesis which leads to the reduction of hormones, moreover, the

reduced levels of testosterone suggest that fipronil may also be interfering with the hypothalamic-pituitary-gonadal axis (Jeng, 2014; Boscolo et al., 2017). Decreased testosterone synthesis may still lead to wrong sexual differentiation in males, because it is a precursor of estrogen biosynthesis (Santos et al., 2015). The 11-kT was at level equal to the control in all treatments between the temperatures tested, indicating no sign of masculinization in the fish in this study. However, more analysis is needed to confirm this result and better understand the fate of 11-kT.

In summary, the results of this study with Sea bass fish revealed that fipronil administered through the food, under the influence of different temperatures, impaired the metabolic and antioxidant responses of exposed animals, as well as influenced the regulation of sex steroid hormones. The relationships between sex hormones and their effects on animal defense mechanisms require further studies to complement and better respond to the effects of fipronil and temperature on these markers. However, these data are of great relevance to understand the effects of environmental variations due to climate change and the influence of the presence of chemical compounds in natural environments.

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CONSIDERAÇÕES FINAIS

4. CONSIDERAÇÕES FINAIS E CONCLUSÃO

Os resultados provenientes dessa pesquisa de doutorado nos permite concluir:

- A exposição à hipóxia levou a uma diminuição no número de transcritos dos receptores GABA e do HIF e nos níveis do neurotransmissor GABA no cérebro das tilápias. Esses resultados foram observados, provavelmente, devido a uma resposta excitatória inicial do sistema nervoso para aumentar a ventilação sob baixa disponibilidade de oxigênio.

- A exposição ao fipronil causou um aumento nos níveis de GABA possivelmente devido ao bloqueio dos receptores GABA-a; tanto o aumento do GABA como o bloqueio dos receptores GABA-a pelo fipronil causaram uma dessensibilização dos receptores GABA-a, o que levou a uma regulação negativa da sua transcrição.

- A exposição ao fipronil por 3 h alterou a resposta do peixe à hipóxia, diminuindo os níveis de RNAm da CAT e causando alterações nos níveis de transcrição dos receptores GABA-a (subunidades γ e β), GABA-B2 e GABA-C, quando comparado ao peixes expostos apenas à hipóxia.

- A transcrição diminuída do mRNA de HIF sugere uma rápida regulação desse fator de transcrição, em menos de 3 h de exposição à hipóxia, onde um pico nos níveis de transcrição gênica é possivelmente observado, mas não é mantido por muito tempo, pois o oxigênio retorna a níveis aceitáveis, contribuindo para otimizar a regulação do HIF em seus níveis de referência.

- A exposição ao fipronil ou exposição à hipóxia são condições deletérias para os peixes, uma vez que causaram alterações significativas nas enzimas antioxidantes e nos níveis de dano às biomoléculas.

- O fipronil mostrou um efeito inibitório sobre a enzima CAT, ao passo que a hipóxia demonstrou estimular as defesas antioxidantes, sendo esses efeitos melhor observado nas brânquias. O efeito desses estressores foram mais evidentes durantes as primeiras horas de exposição, retornando aos valores de controle após 8 horas de exposição, demonstrando ajustes fisiológicos dos órgãos dos peixes à exposição de fipronil e/ou hipóxia.

- Observamos uma diminuição significativa nos níveis de MDA no fígado, sugerindo uma modulação positiva das defesas antioxidantes, um efeito que não foi observado nas enzimas antioxidantes medidas. Alternativamente, estes níveis reduzidos de MDA seriam uma consequência do aumento do seu metabolismo, isto é, por uma indução de ALDH, porém essa é uma hipótese que permanece por esclarecer.

- Os resultados do estudo feito com os peixes *D. labrax* revelaram que o fipronil administrado através do alimento, sob a influência de diferentes temperaturas,

prejudicou as respostas metabólicas e antioxidantes dos animais expostos, bem como influenciou a regulação dos hormônios esteróides sexuais.

Em conclusão, nos resultados demonstraram que a exposição combinada ao fipronil com hipóxia parece aumentar a suscetibilidade do peixe a efeitos fisiológicos negativos, especialmente considerando o maior dano ao DNA em eritrócitos de peixes expostos à exposição mista. A exposição do fipronil administrado na comida em conjunto com variações na temperatura apresentou consequências em termos de estresse oxidativo nos peixes, bem como na regulação de hormôniso sexuais. Além disso, as mudanças observadas neste estudo, sugerem que a presença de agrotóxicos em conjunto com as variações ambientais podem alterar o mecanismo de ação modulatória do GABA. Assim, considerando que em ambientes naturais, os peixes geralmente estão expostos à ação conjunta de estressores ambientais, essa problemática à longo prazo pode causar problemas significativos para os organismos aquáticos, tornando-os mais susceptíveis ao estresse oxidativo e possivelmente modificando os mecanismos adaptativos desenvolvidos pelos peixes para lidar com variações ambientais, como hipóxia e mudanças na temperatura.

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