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Luciana Joner Guerra

RESPOSTAS BIOQUÍMICAS E COMPORTAMENTAIS DE PEIXE-ZEBRA (*Danio rerio*) EXPOSTOS A IMIDACLOPRIDO: AVALIAÇÃO DO DANO OXIDATIVO E PERFIL ANTIOXIDANTE

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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requerido parcial para a obtenção do título de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Prof. Dra. Vania Lucia Loro

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RESUMO

RESPOSTAS BIOQUÍMICAS E COMPORTAMENTAIS DE PEIXE-ZEBRA (*Danio rerio*) EXPOSTOS A IMIDACLOPRIDO: AVALIAÇÃO DO DANO OXIDATIVO E PERFIL ANTIOXIDANTE

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Os pesticidas são um dos maiores poluidores da água e do solo, podendo atingir organismos não alvos, como peixes. A aplicação indiscriminada destes produtos tem levantado sérias preocupações, sendo que um dos países que mais utilizam produtos químicos para controle de pragas agrícolas é o Brasil. O imidacloprido (IMI) é um dos inseticidas mais usados no mundo, caracterizado como um neonicotinóide. Embora já encontrado em vários cursos de água na região sul, são necessários estudos ao seu potencial tóxico. O peixe-zebra (*Danio rerio*), conhecido popularmente como "paulistinha", tem se mostrado um modelo atrativo quando comparado com roedores, podemos citar a facilidade de manutenção, manipulação e a possibilidade de se estudar mecanismos comportamentais e bioquímicos. Nesse contexto, pesquisadores podem ser realizados para avaliar os efeitos da contaminação ambiental. O presente trabalho teve como objetivo avaliar possíveis danos bioquímicos e comportamentais em peixes-zebra expostos por 96 h a 3 diferentes concentrações de IMI (0,15, 15 e 45 μg L^{-1}). Os resultados obtidos indicam que houve a ativação das defesas antioxidantes, uma vez que a atividade da glutationa S-transferase (GST) foi aumentada em cérebros de peixes-zebra quando expostos ao IMI nas concentrações de 0,15 e 45 μg L^{-1}. Os níveis de proteína carbonilada (CP) aumentaram significativamente no IMI 15 e 45 μg L^{-1}. Os teores de tióis não proteicos (NPSH) e os níveis de peroxidação lipídica medidos através da quantificação da formação de malondialdeído (MDA) não apresentaram alterações significativas após a exposição ao IMI nas concentrações testadas. A exposição ao IMI aumenta o tempo de congelamento (freezing), diminui os episódios e o tempo de movimentos erráticos. Além disso, diminui a distância percorrida, as transições e o tempo na área superior nas altas concentrações utilizadas. Em conclusão, os resultados são relevantes uma vez que a exposiçãoao IMI alteram os parâmetros bioquímicos e comportamentais em peixes-zebra.

Palavras-chaves: Dano oxidativo. Peixe-zebra. Imidacloprido. Neonicotinóide.

ABSTRACT

Biochemical and behavioral responses of zebrafish exposed to imidacloprid: Assessment of oxidative damage and antioxidant profile

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Pesticides are the biggest polluters of water and soil and can reach non-target organisms such as fish. Indiscriminate application of products has been a serious concern and Brazil is one of the countries that uses the most chemicals to control agricultural pests. Imidacloprid (IMI) is one of the most commonly used insecticides in the world, such as a neonicotinoid. Although already found in several watercourses in the southern region, studies are needed on its toxic potential. Zebrafish (Danio rerio), popularly known as "paulistinha", has been the target of a program that can be compared with rodents, we can mention the ease of maintenance, manipulation and a possibility to have their rules of behavior and biochemical. In this context, researches may be performe to avaluete effects of environmental contamination. Present indicator may be related to biochemical and behavioral in zebrafish exposed for 96 h in 3 different concentrations of IMI (0.15, 15 and 45 μg L^{-1}). Effect of S-transferase (GST) activation was increases in zebrafish brains when exposed to IMI in the combinations of 0.15 and 45 μg L^{-1}. Carbonylated protein (CP) levels increased significantly in IMI 15 and 45 μg L^{-1}. Nonprotein protein levels (NPSH) and lipid peroxidation levels measured by the quantification of malondialdehyde (MDA) formation were not alter over time after exposure at the concentrations tested. Exposure to IMI increases freezing time, decreases episodes and time of erratic moviment. In addition, it decreases the distance traveled, such as transitions and time in the area above the high turns used. In conclusion, the results are relevant for the exposure of biochemical and behavioral parameters in zebrafish.

Keywords: Zebrafish. Oxidative stress. Imidacloprid. Behavior. Neonicotinoid.

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LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

LISTA DE ABREVEATURAS

IMI – Imidacloprido

TBARS – Espécies reativas ao ácido tiobarbitúrico

AChE – Acetilcolinesterase

GST – Glutationa S-transferase

CP – Proteína carbonil

NPSH – Tióis não proteicos

OF – Organofosforados

DTNB – 5,5'-dithio-bis[2-nitrobenzoic acid]

SUMÁRIO

APRESENTAÇÃO

Esta dissertação aborda assuntos relacionados aos efeitos tóxicos mediados pelo neonicotinóide imidacloprido, avaliando os parâmetros bioquímicos e comportamentais, utilizando o peixe-zebra como organismo modelo. Ela encontra-se estruturada da seguinte forma:

INTRODUÇÃO: Revisão da literatura com caracterização dos temas abordados na dissertação.

MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO: Serão apresentados na forma de artigo científico.

CONCLUSÃO: Comentários gerais sobre os resultados do trabalho.

REFERÊNCIAS: Lista as referências utilizadas na introdução da dissertação.

1. INTRODUÇÃO

1.1. AGROQUÍMICOS

O uso intensivo de diferentes agroquímicos na agricultura moderna está emergindo como uma ameaça para o ambiente e equilíbrio dos ecossistemas aquáticos. São reconhecidos como poluentes graves no meio aquático, com potencial para causar efeitos deletérios na biota, especialmente peixes que ocupam o nível trófico superior (Husak et al., 2014). Áreas agrícolas normalmente se encontram próximas a mananciais, rios e lagos que são utilizados no abastecimento e na manutenção de atividades econômicas. Os agroquímicos utilizados nessas áreas alcancam os corpos de água, por escoamento superficial ou lixiviação, causando danos diretos ao meio ambiente, aos seres vivos que nele habitam e à saúde pública (Zhu et al., 2016). Esses produtos químicos podem causar efeitos adversos, afetando sistemas fisiológicos e comportamentais em organismos não-alvo, como peixes (Erdogan et al., 2011; Karmakar et al., 2016). A exposição constante da biota aquática a substâncias tóxicas lançadas no meio ambiente pode causar múltiplas alterações podendo gerar graves consequências em populações, comunidades ou ecossistemas, dependendo do grau de contaminação e do tempo de exposição (Jesus e Carvalho, 2008).

1.2. NEONICOTINOIDES

A preocupação com o desenvolvimento de neurotoxicidade associada a pesticidas organofosforados (OF) resultou no desenvolvimento de uma nova classe de pesticidas, os neonicotinoides, que compartilham semelhanças com a nicotina. Esta classe de inseticidas compreende pelo menos sete compostos principais que representam perto de 25% do total de vendas de inseticidas em todo o mundo (Bass et al., 2015). A eficácia dos neonicotinoides como pesticidas provem da sua capacidade de atuar como agonistas nos receptores nicotínicos de acetilcolina, uma ação registrada tanto em insetos quanto em mamíferos (Sheets, 2002). Acredita-se que os neonicotinoides tenham reduzida toxicidade em relação aos pesticidas OF, pois se ligam seletivamente aos receptores nicotínicos de acetilcolina no sistema nervoso central de insetos e têm menor ação nos receptores nicotínicos de vertebrados. Porém, estudos demonstram que os neonicotinoides apresentaram um aumento na resistência de insetos-alvo, toxicidade para as abelhas e que são capazes de causar estresse oxidativo e inflamação no sistema nervoso central e no fígado em vertebrados (Duzguner e Erdogan, 2010; Blacquière et al., 2012).

1.3. IMIDACLOPRIDO

O imidacloprido (1- (6-cloro-3-piridilmetil)-N-nitro-imidazolidin-2-ilidenoamina) (IMI) (figura 1) é utilizado em várias formulações para controle de pragas em inúmeras culturas agrícolas no Brasil e no mundo (Pinheiro e Freitas, 2010). Foi o primeiro neonicotinóide introduzido no mercado (Elbert et al., 2008) e o terceiro inseticida mais consumido no Brasil (IBGE, 2015). Devido a sua persistência em ambientes aquáticos e nos solos, o IMI é um potencial risco toxicológico para organismos não alvos, tanto terrestres como aquáticos. Pesticidas neonicotinóides têm sido considerados como uma das principais ameaças à saúde das abelhas que são os insetos que mais estão sendo afetados mundialmente (VETKOV, 2017), No presente estudo foram utilizadas 3 diferentes concentrações, 0,15 μg/L, concentração encontrada em estudos de biomonitoramento ambiental do nosso grupo de pesquisa (DO AMARAL et al., 2018), 15 μg/L e 45 μg/L segundo Crosby et. al (2015), no entando, os dados de toxicidade de imidacloprido em peixes ainda são escassos (Crosby, 2015).

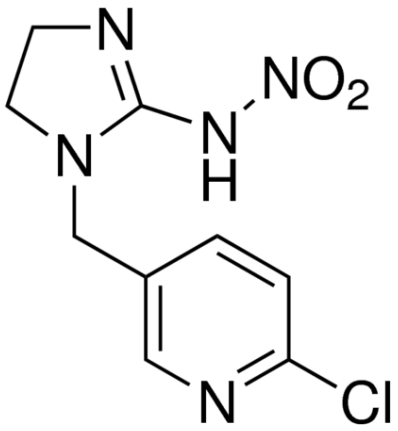

Figura 1. Estrutura química do imidacloprido. (Fonte: Sigma https://www.sigmaaldrich.com/catalog/substance/imidacloprid2556613826141311?lang=pt&region=BR) (Acessado em fevereiro de 2019)

1.4. ESTRESSE OXIDATIVO

Uma das formas de avaliar os danos causados pelos diferentes químicos lançados no ambiente é através de biomarcadores. Biomarcadores são definidos como respostas biológicas adaptativas a estressores, evidenciadas como alterações bioquímicas, celulares, histológicas, fisiológicas ou comportamentais e podem ser usados para vários propósitos, dependendo da finalidade do estudo e do tipo da exposição Em situações de estresse, como na exposição a poluentes ambientais, pode ocorrer o aumento da produção de espécies reativas de oxigênio (ERO) e/ou a diminuição da atividade das defesas antioxidantes, situação caracterizada como

estresse oxidativo (Ferreira et al., 2005). O desbalanço no perfil oxidativo pode estar relacionado com danos no sistema nervoso central (SNC) (Radak et al., 2011). As ERO são produzidas na mitocôndria durante o metabolismo aeróbico normal, entretanto se não houver produção aumentada de espécies reativas o organismo consegue manter o equilíbrio entre pró-oxidantes e antioxidantes, sem maiores danos ao organismo. Porém, quando o dano oxidativo ocorre, ele pode afetar lipídios, proteínas e DNA, podendo desencadear eventos como a morte celular (Valko et al., 2007; Birnie-Gauvin et al., 2017). Sistemas biológicos possuem antioxidantes que minimizam os efeitos do aumento de ERO, como as enzimas superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx), e defesas não enzimáticas como a glutationa reduzida (GSH) (Valko et al., 2007; Dal Santo, 2014).

1.5. PEIXE-ZEBRA

O peixe-zebra (*Danio rerio*) é uma espécie tropical de água doce conhecida popularmente como “paulistinha”, é um modelo amplamente utilizado em estudos toxicológicos devido às suas características, como grande similaridade com humanos, apresentando conservação genética de cerca de 70% em comparação com genes humanos e respostas de comportamento complexo (Levin e Cerutti, 2008; Quadros et al, 2016). O pequeno tamanho e manutenção relativamente fácil do peixe-zebra também oferece benefícios econômicos e logísticos sobre os modelos de mamíferos.

Figura 2. Exemplar de peixe-zebra (*Danio rerio*)
https://www.unochapeco.edu.br/noticias/pesquisa-inovadora-e-realizada-na-unochapeco
(Acessado em fevereiro de 2019)

Neste estudo, utilizaremos o peixe-zebra como organismo modelo para determinar os efeitos do imidacloprido na função neurocomportamental e toxicológica. De acordo com estudos já realizados com invertebrados, o imidacloprido (IMI) altera a atividade da enzima acetilcolinesterase (AChE) (Crosby, 2015; Ge, 2015; Topal, 2017) e é capaz de causar estresse oxidativo e inflamação no sistema nervoso central, brânquias e no fígado de vertebrados (Duzguner, 2010; Vieira, 2018).

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo desta dissertação foi avaliar as alterações promovidas pelo neonicotinóide imidacloprido sobre parâmetros comportamentais e bioquímicos em peixe-zebra.

2.2 OBJETIVOS ESPECÍFICOS

- Identificar quais concentrações de imidacloprido causaram alterações no perfil oxidativo de peixe-zebra;

- Determinar a atividade da enzima acetilcolinesterase no cérebro e correlacionar os resultados com os parâmetros comportamentais apresentados.

- Determinação de parâmetros oxidativos não enzimáticos como carbonil e TBARS de cérebro;

- Determinar padrão antioxidante não enzimático como os tióis não proteicos;

- Determinar a atividade da enzima GST em cérebro;

- Avaliar quais respostas comportamentais poderiam ser responsáveis pela exposição de diferentes concentrações de imidacloprido.

3. MANUSCRITO

Biochemical and behavioral responses of zebrafish exposed to imidacloprid: Assessment of oxidative damage and antioxidant profile

Luciana Joner Guerra, Aline B. Amaral, Vanessa A. Quadros, Tiago L. Fiuza, Vania L. Loro

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Biochemical and behavioral responses of zebrafish exposed to imidacloprid: Assessment of oxidative damage and antioxidant profile

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ABSTRACT

Imidacloprid (IMI) is an insecticide used worldwide, characterized as a neonicotinoid that could cause toxicity in non-target organisms. Zebrafish (*Danio rerio*) is a model organism widely used in scientific research in different areas of knowledge such as behavioral studies, biochemical parameters as well as neurotoxicity research. Thus, the objective of this study was to identify the potential risk of IMI in zebrafish exposed for 96 h at 3 concentrations of IMI (0.15, 15 and 45 μg L^{-1}). The results indicate that antioxidant defenses were activated because glutathione S-transferase (GST) activity increased in zebrafish brain when exposed to IMI at concentrations of 0.15 and 45 μg L^{-1}. The levels of carbonyl protein (CP) increased significantly in IMI 15 and 45 μg L^{-1}. The levels of non-protein thiols (NPSH) and thiobarbituric reactive substance (TBARS) did not show significant changes after exposure to IMI at concentrations tested. In addition, IMI increases freezing time and decreases episodes and time of erratic movements. Besides that, decreases distance traveled, transitions and time in the top area at the high concentrations used. In conclusion, results are relevant because they present the behavioral and toxicity effects of IMI together and can cause oxidative damage in the zebrafish brain.

Keywords: Zebrafish. Oxidative stress. Imidacloprid. Behavior. Neonicotinoid

Introduction

Neonicotinoid insecticides comprise one of the most important classes of synthetic pesticides used in agricultural practices. These pesticides are used to protect crops against insects with piercing-sucking mouthparts, representing around 25% of total insecticide sales worldwide [1, 2]. Neonicotinoids increase the resistance of target insects, and is also highly toxic to bees [3, 4]. The extensive use of pesticides as imidacloprid (IMI) in agriculture is a global problem due to ecological impacts in non-target organisms, like fish and birds [5-6]. IMI (1-(6-chloro-3-pyridylmethyl)-N-nitroimadazolidin-2-ylide neamine) is a very common insecticide used for controlling sucking insects in farming [7], as well as leafhopper, aphids, and thrips on paddy, vegetables and other cultures [8]. The active principle of IMI acts as an agonist of nicotinic acetylcholine receptor (nAChR) and frequently may impair the central nervous system homeostasis. Additionally, IMI acts a potential surface-water contaminant when residual of IMI metabolites enter water bodies from crops, contaminate soil, also by accidental spills or spray drift, resulting in local pollution [9, 10]. Despite the increasing use of IMI, there are few studies on the effects of IMI on non-targeted organisms such as algae, bacterium, earthworm, and fish [11, 12].

Zebrafish (*Danio rerio*) is widely used in toxicological studies due to its advantageous characteristics, such as genetic conservation (around 70% when compared to the human genes) [13, 14], and evolutionarily conserved neurotransmitter systems [13, 15-18]. Concerning the relation between fish behavior and biochemical parameters there are few studies aiming to understand the neurological mechanisms associated with behavioral parameters in theis species, which will be important in selecting potential biomarkers involved in toxicological mechanisms [14, 19]. The effects of neonicotinoids on behavioral and biochemical parameters in vertebrates have not yet been well characterized and assumptions about their safety and damage were made in the absence of a thorough investigation. Thus, we aimed to evaluate the potentially harmful

effects of IMI on Zebrafish considering biochemical and behavioral parameters to verify the use of this species as bioindicator of contamination in toxicology studies.

MATERIAIS AND METHODS

Animals

Adult zebrafish (4-5 months-old) of short fin wild-type (wt), were acquired in local commercial suppliers (Hobby Aquários, RS, Brazil). The proportion used for the experiments was a 50:50 (male: female). Fish were maintained with a maximum density of four fish per liter in a 20 L tank (27±1ºC), and the animals were acclimatized for 15 days in 40 L aquariums prior to the experiments. Water was kept under mechanical, biological, and chemical filtration, dechlorinated with AquaSafe ™ (Tetra, VA, USA). Illumination was provided by a photoperiod cycle, 14h/10h, light/dark respectively, using fluorescent light tubes. Fish were fed three times daily with Alcon BASIC ™ flake fish food (Alcon, Brazil). This experiments fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number:1777051118).

Preparation and Administration of the IMI

IMI was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the solution was prepared in ultrapure water. Exposure to IMI was performed at three different concentrations (0.15, 15 and 45 μg L^{-1}) prepared from stock solutions (100 mgL^{-1} and 1059 mg L^{-1}). The concentrations of IMI were chosen based on environmental analyses [20] and previous studies [21].

Water Analysis

Water samples were collected at the beginning, middle and end of experimental period to perform the quantitative analysis of IMI. Analysis of the water samples was performed by Solid Phase Extraction (SPE) and using a ultra-high efficiency liquid chromatography coupled in tandem with mass spectrometry. For the SPE procedure, Oasis® HLB cartridges were used, and samples were percolated and eluted with the acidified mixture of the MeOH:MeCN (1:1, v / v) solvents. Prior to the chromatographic injection, samples were diluted twice in ultrapure water [22]. Results were showed in table 1.

Experimental Design

After the acclimation period, groups of 12 fish were transferred to 20 L tanks for exposure. Four groups were tested: control, IMI 0.15 μg L^{-1}, IMI 15 μg L^{-1} and IMI 45 μg L^{-1}. Fish were exposed to IMI on the first day and remained in the tank with the agrochemical for 96 hours. After the exposure period, behavioral tests were performed and then animals were anesthetized in water 4ºC and euthanized by cervical section.

BEHAVIORAL MEASUREMENTS

Novel tank test

All experiments were performed between 09:00 am and 4:00 pm. During the exposure protocol, the animals were handled with all precautions to minimize stress during the experiments. Behaviors were recorded using with a webcam connected to a laptop at a rate of 30 frames/s, and subsequently the behavioral parameters were analyzed using automated video tracking system ANY-maze™ (Stoelting CO, USA).

We used the novel tank test to analyze both locomotor and exploratory activity of zebrafish in a novel environment, which may reflect habituation to novelty stress [23]. At the

end of exposure fish were placed individually in a novel apparatus (25 cm length × 15 cm height × 11 cm width) (Figure 1) divided into three horizontal portions (bottom and top). The tank was filled with 2 L home tank water and the swimming behavior was recorded. Locomotor activity was measured by distance traveled (m) and angular velocity (◦/s). Fear/anxiety-related behaviors were determined by quantifying the number and duration (s) of freezing bouts as well as the number and duration (s) of erratic movements. Vertical exploration was assessed by the following endpoints: number of entries and time spent (s) in the bottom area, latency (s) to enter the top, number of entries and time spent (s) in the top area. The habituation profile was evaluated by assessing the number of entries and time spent (s) in top during the 6-min trial [24].

BIOCHEMICAL PARAMETERS

Tissue preparation

After the behavioral assays, fish were previously anesthetized in ice water at 4°C and subsequently euthanized by decapitation and the brain was dissected on ice and transferred to microtubes for storage at -80 °C. For each independent sample, two brains were pooled and homogenized in 300 μL of Tris-HCl 50 mM buffer, pH 7.4. Samples were centrifuged (3000 x *g* for 10 min, -4°C) and the supernatant was removed for further testing. All tests were performed in duplicate.

Lipid peroxidation

The lipid redox status of the samples was estimated by thiobarbituric reactive substance (TBARS) production [25]. Briefly, 150 μL of 10% TCA was added in 75 μL of the homogenized brain (70-100 μg protein) and subsequently centrifuged at 10,000 x *g* for 10 min. After 100 μL of the supernatant was added to 100 μL of 0.67% thiobarbituric acid (TBA, 4,6-

dihydroxypyrimidine-2-thiol) and heated for 30 min at 100 °C. Determination of TBARS levels was performed with malondialdehyde (MDA) reaction, in a microplate reader, at 532 nm. Data were expressed as nmol MDA/milligram protein and MDA was utilized as standard. For more details see Nunes et al. [26].

Carbonyl protein (CP) levels was determined using the method described elsewhere [27]. We used 200 μL of the soluble protein blended in 10 mM DNPH in 2 N hydrochloric acid. In a dark environment, samples were incubated for 1h and then 0.15 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8 containing SDS 3.0%), 0.5 mL of heptane (99.5%) and 0.5 mL of ethanol (99.8%) were added and kept under continuous agitation for 40 s, finally centrifuged for 15 min at 3000g. After isolation, the protein was washed twice by resuspension in ethanol/ethyl acetate (1:1) and resuspended in 0.25 ml of denaturing buffer. Data were measured in a spectrophotometer at 370 nm in a microplate reader, and calculated using the molar extinction coefficient of 22,000 M/cm. Total carbonylation was expressed as nmol carbonyl/milligram protein. For more details see Muller et al. [28].

Antioxidant parameters

Glutathione S-transferase (GST) activity was measured according to the literature [29], using 1mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, 10 mM reduced glutathione, 20 mM potassium phosphate buffer (pH 6.5) and 10 μL of tissue homogenates (40-60 μg protein). Enzyme activity was measured by variations in absorbance at 340 nm using a molar extinction coefficient of 9.6 mM/cm in a microplate reader. GST activity was determined according to the amount of enzyme required to catalyze the 1 mol CDNB conjugate with GSH/min at 25°C. Results were expressed in micromole GS-DNB/min/milligram protein.

For non-protein thiols quantification, we utilized an aliquot of supernatant (100 μL) mixed with 100 μL of 10 % trichloroacetic acid (TCA) and later centrifuged (3000 x *g* for 10

min at 4 °C). Supernatants (60–80 µg protein) were mixed with 5,5'-dithio-bis[2-nitrobenzoic acid] DTNB (0.01 M dissolved in ethanol) and the intense yellow color developed was measured at 412 nm after 1 h [30]. Results were expressed as nmol SH/mg of protein.

Acetylcholinesterase (AChE) activity

AChE activity was measured as described previously [31] with some modifications. Aliquots of supernatants (10 µL) were pre-incubated at 30°C for 2 min with 0.1 M phosphate buffer, pH 7.5 and 1 mM DTNB. The reaction was started by the addition of acetylthiocholine (1 mM) and kinetic reation readings were made at 412 nm. The activity was expressed as µmol of acetylthiocholine (ASCh) hydrolyzed/mg protein/min.

Protein quantification

Protein was determined using the Coomassie blue method and bovine serum albumin as standard [32]. Samples were run in duplicate and the absorbance was measured at 595 nm.

Statistical analyses

Normality of data and homogeneity of variances were analyze using Holm-Sidak's multiple comparisons test and Bartlett's test, respectively, respectively. Parametric data were expressed as mean ± standard error of mean (S.E.M) and analyzed by one-way analysis of variance (ANOVA). Results were expresses as a means ± standard error of the mean (S.E.M.). Data were significant when $p \leq 0.05$.

RESULTS

BEHAVIORAL ANALYSES

Exposure to IMI alters locomotion

IMI exposure decreased the distance traveled at 45 µg L^{-1} when compared to control ($F_{(3.52)}$ =13.29, p<0.0001), (Fig. 2a). All concentrations decreased both transitions and time spent in top area as compared to control ($F_{(3.52)}$=11.82, p=<0.0001; $F_{(3.50)}$=12.67, p=<0.0001, respectively), (Fig. 2b). Conversely, the Absolute turn angle, maximum speed, and the latency to enter the top did not change.

Freezing and erratic movements

At 45 µg L^{-1} a significant decrease in the episodes of erratic movements was observed when compared to control ($F_{(3.52)}$=10.62, p=<0.0001) (Fig. 3a). Duration of erratic movements decreased in 45 µg L^{-1} of IMI compared to the control ($F_{(3,52)}$=5.491, p=0.0024) (Fig. 3b). Freezing episodes increased at 45 µg L^{-1} of IMI compared to control ($F_{(3.52)}$=8.389, p=*0*.0001) (Fig. 3c). At 45 µg L^{-1}, IMI increased freezing duration compared to control ($F_{(3.52)}$=10.62, p=<0.0001) (Fig. 3d).

Biochemical analyses

Imidacloprid exposure alters antioxidant parameters and lipid peroxidation in the brain

Concentrations of 0.15 µg L^{-1} and 45 µg L^{-1} increased GST activity in relation to the control ($F_{(3.20)}$=1.216, p=0.00012 (Fig. 4a). Although CP levels increased at 15 µg L^{-1} and 45 µg L^{-1} compared to control ($F_{(3.20)}$=1.349, p<0.0001) (Fig. 4a), NPSH and TBARS did not changesignificantly (Fig. 4b).

AChE activity

Animals exposed to 15 µg L^{-1} and 45 µg L^{-1} IMI showed lower AChE activity compared to control ($F_{(3.22)}$=1.266, p=<0.0001) (Fig. 5).

DISCUSSION

Because the toxic effects of IMI are not fully understood, here we verified how different IMI concentrations affect behavioral profile of zebrafish, as well as the putative biochemical mechanisms involved in such responses. To our knowledge, there are few data regarding the toxic effects of IMI on the central nervous system of fish. This study reported for the first time that IMI induces oxidative damage in the zebrafish brain, suggesting that oxidant parameters may play a role, at least partially, with the behavioral effects observed here.

Regarding the behavior pattern, IMI at low concentrations (0.15 and 15 µg L^{-1}) changed the vertical activity of fish by reducing both time spent and transitions to the top area. However, when exposed to the highest concentration (45 µg L^{-1}), the animals showed impaired locomotion and froze in the bottom area of the tank, with decreased erratic movements. These data suggest a dual effect of IMI on zebrafish behavior. Basically, lower concentrations affect vertical activity without changing locomotion, while 45 µg L^{-1} could act as a stressor agent, causing hypolocomotion as a putative symptom of toxicity. Animals exposed to toxic agents, such as pesticides, change their behavior as a first response to an acute stressor [33]. Our results corroborate with previous findings, showing that when fish are exposed to toxic agents, they spend more time in the bottom of the aquarium with a reduced vertical swimming activity [34].

A common response of fish against pesticides toxicity is the increase of oxidative damage as lipid peroxidation and formation of carbonyl proteins due to oxidative stress [35]. These parameters serve as important biomarkers to verify the effects of pesticides, as IMI [36]. Carbonylation of proteins is an indicative of oxidative damage to proteins. Increased CP levels

were observe in fish exposed to 15 and 45 μg L^{-1} IMI indicating that high concentrations induce protein damage. Conversely, the increase of GST activity suggests an activation of the antioxidant defenses, even though they were not sufficient to prevent carbonylation of proteins [35]. The oxidative damage caused by IMI in zebrafish brain occurs through protein oxidation and not due to lipid peroxidation. On the contrary, when *Prochilodus lineatus* were exposed to IMI, brain showed different results. Lipid peroxidation increased and there was no statistical difference in relation to carbonyl levels [35]. In the present study IMI did not change MDA production as shown [37].

AChE is the major enzyme involved in the cleavage of the neurotransmitter acetylcholine in choline and acetate [38]. Fish exposed to 15 and 45 μg L^{-1} IMI showed a decreased AChE activity, indicating impaired cholinergic activity. These novel findings suggest that the modulation of cholinergic signaling could be involved, at least in part, with the behavioral phenotypes of locomotion and motricity in zebrafish as AChE was inhibited by the action of IMI, this inhibitory response may influence the behavioral responses measured here. Similar results to the decrease of AChE were found in previous studies that used the same fish as experimental model [39]. Although more studies are needed to associate how cholinergic signaling correlate with oxidative stress in zebrafish brain, there is a clear relationship between the inhibition of AChE with behavioral changes [40].

Overall, we demonstrated in the present study that the behavior of Zebrafish is affected by IMI as well the AChE activity. Probably IMI exposure is able to modulate cholinergic system causing behavioral impairment. The results of protein carbonyl configure the oxidative damage to proteins due to IMI exposure. In addition results obtained reinforce the increasing utility of this aquatic species in studies related to toxicology and behavior.

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Figure captions

Fig. 1. Schematic representation of the experimental protocol. The animals were exposed to IMI for 96 hours at 3 different concentrations (0.15, 15 and 45 μgL^{-1}) after the exposure period the animals were tested behaviorally and the brain was withdrawn for neurochemical and oxidative stress analyzes.

Fig. 2. The influence of exposure to IMI on responses in the novel tank in zebrafish. (a) locomotor parameters, (b) exploration parameters. Date were expressed as means ± S.E.M and analyzed by one–way ANOVA by Newman-Keuls multiple comparisons test. (****p<0.0001, n= 12-14 per group).

Fig. 3. Aversive behaviors triggered by IMI in zebrafish. The defensive reactions (freezing and erratic movements). The scores obtained per group were depicted as representative heat maps (a,b) episodes an number of erratic movement, respectively (c,d) episodes an time of freezing, respectively. Date were expressed as means ± S.E.M and analyzed by one–way ANOVA by Newman-Keuls multiple comparisons test. (****p=0.00012, ****p<0.0001, n= 12-14 per group).

Fig. 4. Effects of exposure to IMI on oxidative estress in brain. (a) activity GST and levels of TBARS. Date were expressed as means ± S.E.M and analyzed by one–way ANOVA by Newman-Keuls multiple comparisons test. (****p<0.0001, ****p=0.0001, n= 12-14 per group).

Fig. 5. Brain AChE activity in zebrafish after the exposure to IMI. (a) activity GST and levels of TBARS. Date were expressed as means ± S.E.M and analyzed by one–way ANOVA by Newman-Keuls multiple comparisons test. (****p<0.0001, n= 12-14 per group).

Fig 6. Imidacloprid modifies behavioral parameters, oxidative stress-related parameters and AChE in zebrafish brain. Representative effects observed are schematically shown.

Table 1. Quantification of IMI levels in water of experimental protocol

Figure 1

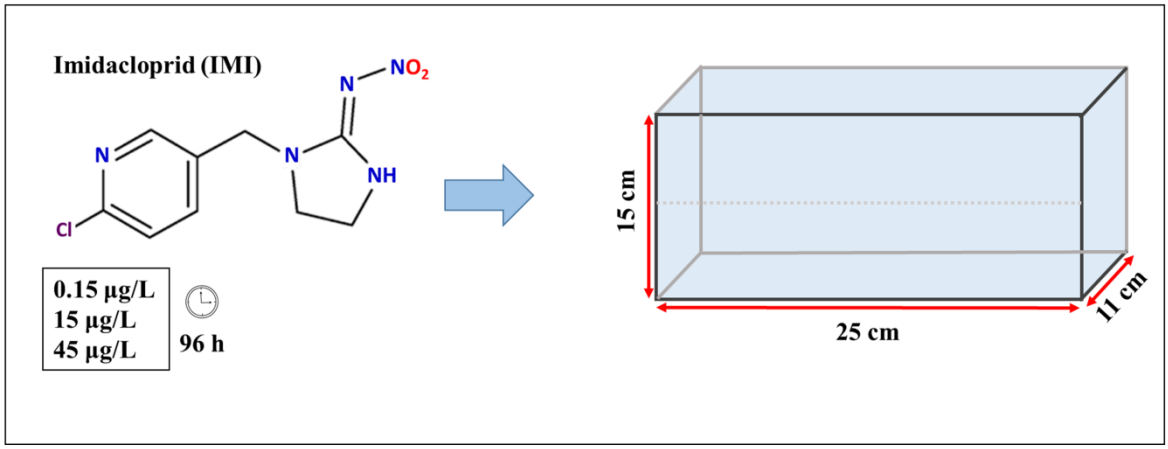

Figure 2

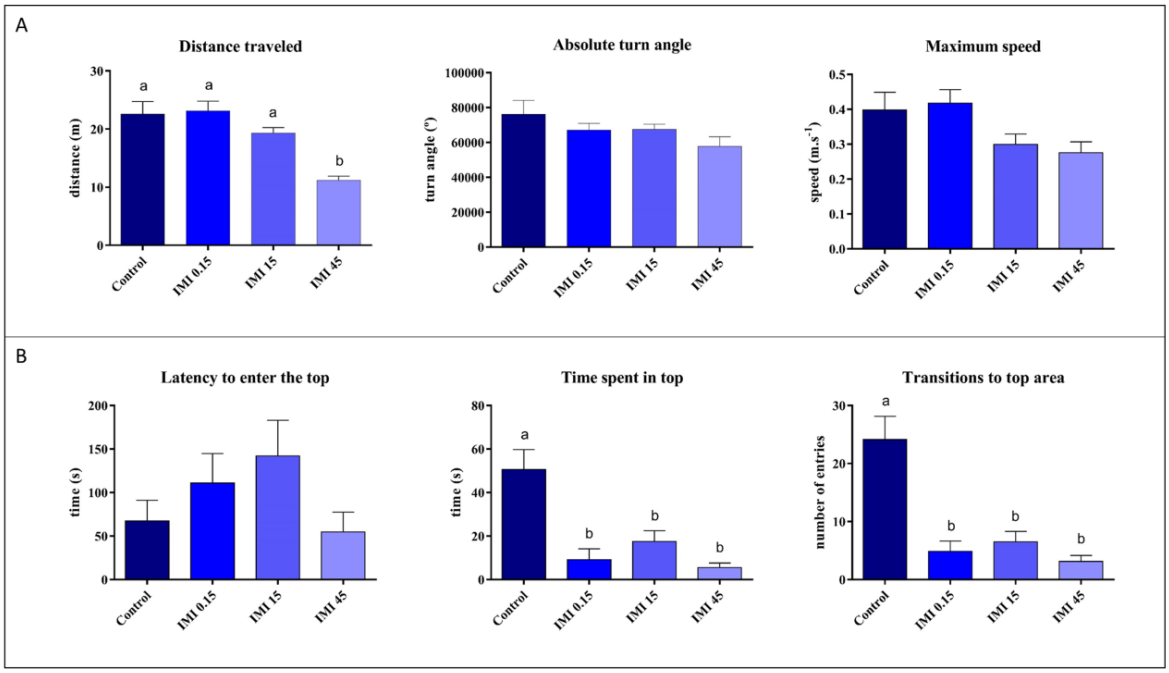

Figure 3

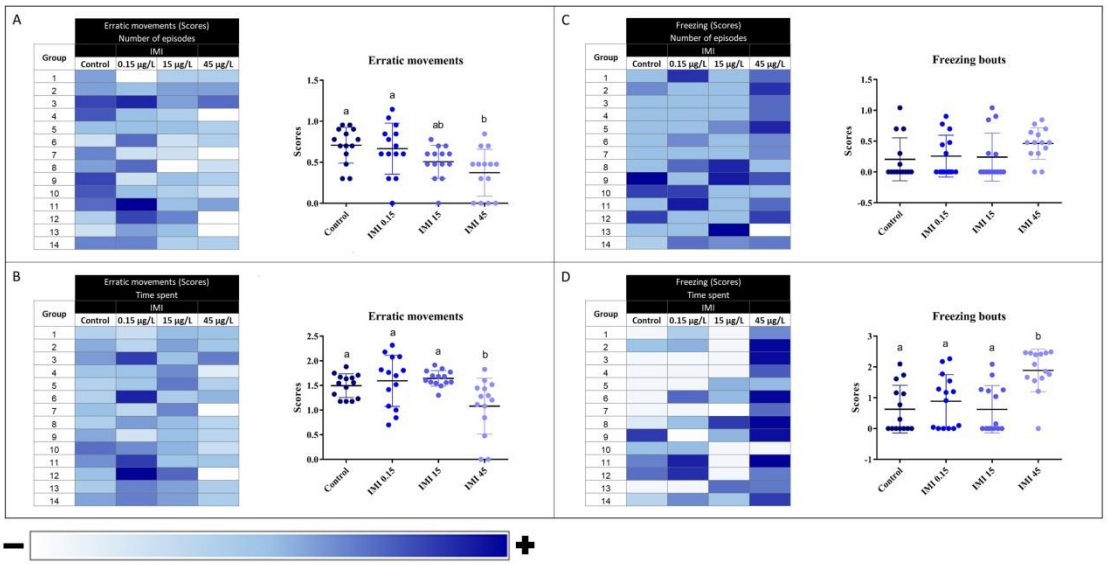

Figure 4

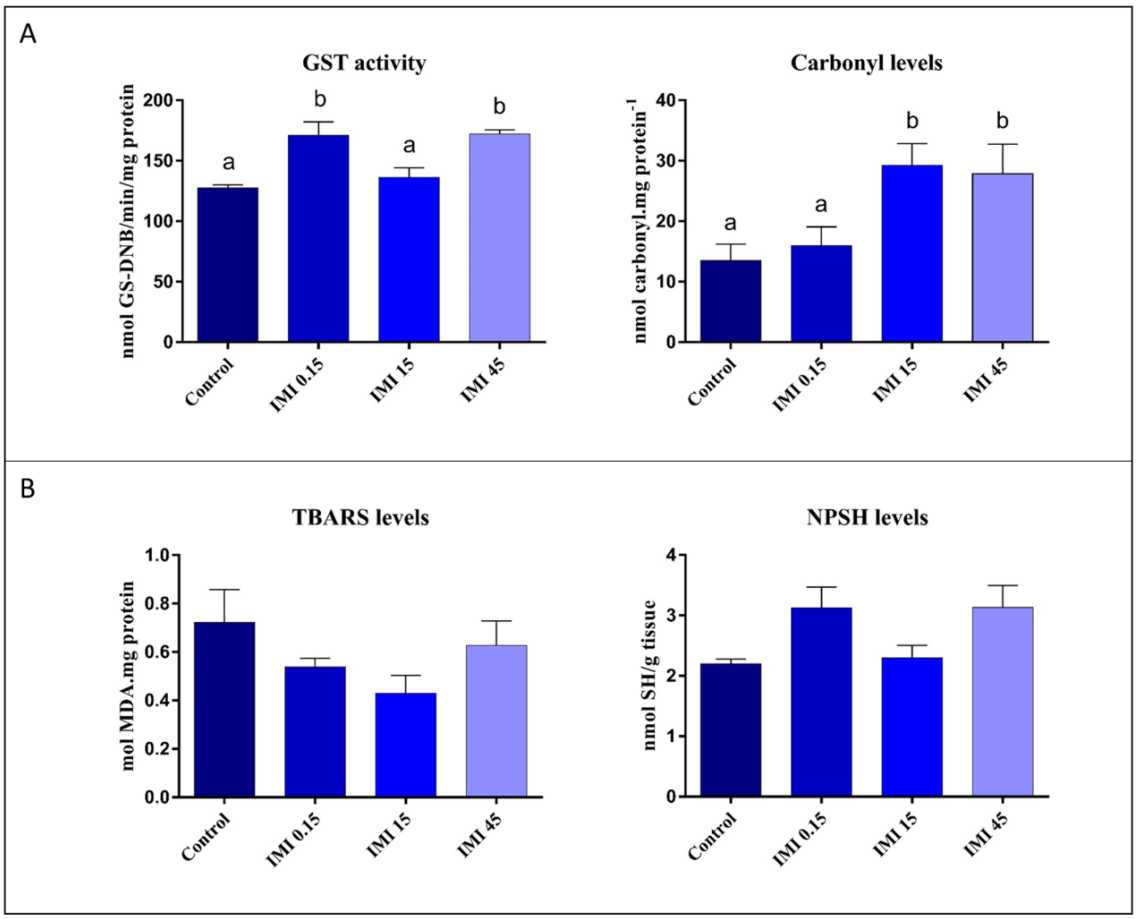

Figure 5

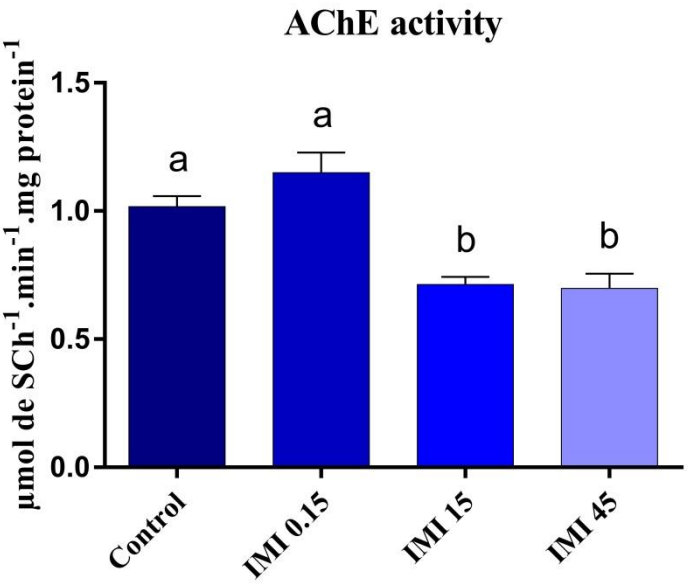

Table 1.

Nominal concentration	Sampling time	[Measured]	% after 96h
IMI 0.15 µgL^{-1}	0h	0.15 µgL^{-1}	
	96h	0.14 µgL^{-1}	93.33%
IMI 15 µgL^{-1}	0h	14 µgL^{-1}	
	96h	11.95 µgL^{-1}	85.35%
IMI 45 µgL^{-1}	0h	37.17 µgL^{-1}	
	96h	35.89 µgL^{-1}	81.37%

*Limit of detection (LOD): 0.006 µgL^{-1}; limit of quantification: 0.020 µgL^{-1}.

4. CONCLUSÕES

As respostas bioquímicas e comportamentais induzidas pelo neonicotinóide imidacloprido são dependentes da concentração que foram expostos. Esta conclusão pode ser sustentada pelos seguintes dados:

- Todas as concentrações testadas causaram alterações no perfil oxidativo.
- A AChE foi inibida nas concentrações maiores e nessas mesmas concentrações os parâmetros comportamentais foram mais afetados.
- Houve um aumento da proteína carbonil enquanto o TBARS não foi afetado.
- Não houve alteração nos tióis não proteicos.
- Ocorreu a ativação da enzima GST.
- No geral a exposição alterou diversos parâmetros locomotores do peixe zebra, mesmo nas concentrações mais baixas.

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Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Parâmetros comportamentais e bioquímicos em peixe zebra (*Danio rerio*) expostos aos pesticidas paraquat e imidacloprido", protocolada sob o CEUA nº 1777051118, sob a responsabilidade de **Vania Lucia Loro** e equipe; Talise Müller, Luciana Joner Guerra, Tiago da Luz Fluza; Vanessa Andreatta de Quadros - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de **11/08/2016**. Uma emenda foi adicionada ao projeto para a repetição do experimento exceto a parte da dieta, utilizando-se o neonicotinóide imidacloprido (IMI). A emenda foi **aprovada** na reunião de 07 de março de 2017.

We certify that the proposal "biochemical and behavior parameters of zebrafish (Danio rerio) exposed to pesticides paraquat and imidaclopride". The experimental protocols used 120 zebrafish for each pesticide under protocol number 1777051118, under the responsibility of **Vania Lucia Loro** *and team*; Talise Müller, Luciana Joner Guerra, Tiago da Luz Fiuza and Vanessa Andreatta de Quadros - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of **11/08/2016**. One addition was added to protocol, using the neonicotinic pesticide imidaclopride. The changes was approved by CEUA/UFSM in the meeting of 07/03/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 01/2016 a 03/2018 Área: Bioquímica E Biologia Molecular

Origem:	Biotério externo	sexo:	Machos de fêmeas	idade:	3 meses	N:	240
Espécie:	Peixe						
Linhagem:	Danio rerio			Peso:	0,4 a 0,6 g		

Resumo: Com o aumento de atividades industriais, mineração e também atividades agrícolas, diversos contaminantes são liberados no meio ambiente. Estes contaminantes podem ser provenientes do uso de defensivos agrícolas e também podem ser liberados para o meio ambiente de várias formas através da lixiviação, contaminação do solo e outros meios. O presente estudo visa testar 2 classes de pesticidas que são utilizados em lavouras do Brazil. Os peixes serão expostos durante 96h á concentrações estabelecidas dos dois compostos separadamente. Em cada teste serão utilizados 120 peixes da espécie Danio rerio, também conhecido como peixe zebra. O modelo peixe zebra foi escolhido devido a praticidade do manejo, baixo custo de manutenção e também para juntar as análises bioquímicas testes de comportamento.

Local do experimento: Os experimentos envolvendo peixes serão realizados no biotério de experimentação do laboratório de fisiologia de peixes sobre responsabilidade do professor Bernardo Baldisserotto e professora Vania Lucia Loro.

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

Santa Maria, 07 março de 2017

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria