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Giovana Rolim de Oliveira

EFEITOS COMPORTAMENTAIS E NEUROQUÍMICOS DO CARBOFURANO EM PEIXES-ZEBRA

Porto Alegre
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Farmacologia e Terapêutica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de mestre em Farmacologia e Terapêutica.

Orientador: Prof. Dr. Angelo Piato
Coorientadora: Profa. Dra. Rosane Gomez

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RESUMO

O carbofurano (CF) (pesticida carbamato) é amplamente utilizado na agricultura e encontrado como contaminante no meio ambiente e possui alta toxicidade. Ele atua como inibidor da acetilcolinesterase, enzima que degrada a acetilcolina, um dos principais neurotransmissores. Esse acúmulo de acetilcolina resulta na síndrome colinérgica que acarreta danos à saúde humana. No entanto, dados sobre os efeitos do CF no sistema nervoso central ainda são escassos. Este estudo teve como objetivo investigar os efeitos da exposição ao CF em parâmetros comportamentais e neuroquímicos em peixes-zebra adultos. Os animais foram expostos por 96 horas a diferentes concentrações de CF (5, 50 e 500 µg/L) e submetidos a avaliações comportamentais no teste do tanque novo (NTT) e no teste de preferência social (SPT). Posteriormente, foram avaliados marcadores neuroquímicos associados ao estresse oxidativo e os níveis de acetilcolinesterase. No NTT e SPT, o CF não alterou os parâmetros comportamentais avaliados. Além disso, o CF não afetou a atividade da AChE, tióis não proteicos (NPSH) e substâncias reativas ao ácido tiobarbitúrico (TBARS) no encéfalo dos peixes-zebra. Este estudo demonstrou que o CF não afetou o comportamento e o status oxidativo em peixes-zebra nas condições estudadas. No entanto, mais pesquisas são necessárias em relação à exposição ambiental desse composto em organismos não-alvo.

Palavras-chave: Carbofurano, pesticidas, comportamento, *Danio rerio*, peixe-zebra, estresse oxidativo

ABSTRACT

Carbofuran (CF) (carbamate pesticide) is widely used in agriculture and found as a contaminant in the environment, possessing high toxicity. It acts as an inhibitor of acetylcholinesterase, an enzyme that degrades acetylcholine, one of the main neurotransmitters. This accumulation of acetylcholine results in cholinergic syndrome, which causes damage to human health. However, data on the effects of CF on the central nervous system are still scarce. This study aimed to investigate the effects of CF exposure on behavioral and neurochemical parameters in adult zebrafish. The animals were exposed for 96 hours to different concentrations of CF (5, 50, and 500 µg/L) and subjected to behavioral assessments in the novel tank test (NTT) and the social preference test (SPT). Subsequently, neurochemical markers associated with oxidative stress and acetylcholinesterase levels were evaluated. In the NTT and SPT, CF did not alter the assessed behavioral parameters. Furthermore, CF did not affect the AChE activity, non-protein thiols (NPSH), and thiobarbituric acid reactive substances (TBARS) in the zebrafish brain. This study demonstrated that CF did not affect behavior and oxidative status in zebrafish under the studied conditions. However, more research is needed regarding the environmental exposure of this compound in non-target organisms.

Keywords: Carbofuran, pesticides, behavior, *Danio rerio*, acetylcholinesterase, oxidative stress

LISTA DE SIGLAS E ABREVIATURAS

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais

ANVISA – Agência Nacional de Vigilância Sanitária

IA – Ingrediente ativo

ha – Hectare

kg/ha – Quilos por área

L – Litro

µg/L – Micrograma por litro

µg/kg – Micrograma por quilo

mg – Miligrama

PARA – Programa de Análise de Resíduos

ACh – Acetilcolina

AChE – Acetilcolinesterase

NPSH – Tióis não-proteicos

TBARS – Substâncias reativas ao ácido tiobarbitúrico

mg/dL – Miligrama por decilitro

KOC – Coeficiente de partição de carbono orgânico

NTT – *Novel tank test*

SPT – *Social preference test*

SUMÁRIO

1. INTRODUÇÃO

1.1 Pesticidas

Há cerca de 85 anos era criado o primeiro pesticida moderno, o composto organoclorado dicloro-difenil-tricloroetano (DDT), criado na Segunda Guerra Mundial com finalidade de uso como inseticida (Ribeiro e Pereira, 2016). Desde então, diversos outros compostos foram lançados no mercado, contribuindo para que a produção agrícola dê conta da demanda alimentar mundial (Ruomeng et al., 2023; United Nations, 2022.). Os pesticidas são descritos como qualquer substância ou mistura de substâncias químicas e biológicas destinadas a repelir, destruir e controlar qualquer praga ou regular o crescimento das plantas (FAO and WHO, 2022). No Brasil, o Ministério da Agricultura, Pecuária e Abastecimento (MAPA), o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais (IBAMA) e a Agência Nacional de Vigilância Sanitária (ANVISA) são os órgãos com competência para realizar a fiscalização dos produtos (Albuquerque et al., 2016). Desde 2008, o Brasil está entre os maiores consumidores de pesticidas do mundo. Entre os anos de 2012 e 2014, a comercialização de pesticidas cresceu de 823.226 para 914.220 toneladas (INCA, 2021). O relatório de Indicadores de Desenvolvimento Sustentável (INCA, 2021) indicou um aumento do consumo de ingredientes ativos (IA) por área plantada (hectare, ha), de 3,2 kg/ha para 6,7 kg/ha, no período de 2000 a 2014, o que representa mais de 100% de aumento no consumo. O PARA, Programa de Análise de Resíduos de Agrotóxicos em Alimentos (um programa criado em 2001 com o objetivo de avaliar continuamente os níveis de resíduos de pesticidas nos alimentos que estão sendo comercializados no Brasil) realizou em 2018 uma análise de 4616 amostras de alimentos variados de diferentes regiões e dentro dessas amostras, 23% foram consideradas insatisfatórias para consumo pois apresentaram ou ingrediente ativo acima do limite estabelecido pela ANVISA, ou ingrediente ativo proibido para a cultura em que foi destinado ou ingrediente ativo proibido no Brasil. Os pesticidas mais detectados foram imidacloprido, tebuconazol, carbendazim, piraclostrobina e ditiocarbamatos (Programa de Análise de Resíduos de Agrotóxicos em Alimentos (PARA), 2019). Apesar da ampla utilização, faltam informações sobre a quantidade e tipo de produto utilizado por unidade da Federação, o que dificulta a implantação de uma política pública de prevenção de intoxicações e de contaminação ambiental (INCA, 2021).

O uso desses compostos em larga escala tem implicado em uma série de questionamentos, devido ao potencial impacto em organismos não-alvo presentes no ecossistema, incluindo os seres humanos (Dutra and Ferreira, 2017; Neves et al., 2020). Os efeitos da exposição aos pesticidas em populações não-alvo já são estudados há anos. Podemse destacar alterações em eixos neuroendócrinos (Kumar et al., 2023), malformações congênitas (Dutra e Ferreira, 2017), alterações na qualidade espermática (Abell et al., 2000) e doenças cardiovasculares (Zago et al., 2020). Importante ressaltar que a etiologia de doenças neurodegenerativas, como Parkinson e Alzheimer, tem sido associada à exposição de pesticidas (Baltazar et al., 2014; Kamel et al., 2007; Parrón et al., 2011; Tang, 2020). Também é importante a associação entre a exposição a pesticidas e ansiedade e depressão (Cancino et al., 2023; Freire and Koifman, 2013; Wu et al., 2023; Zanchi et al., 2023). No Brasil, já existem estudos que demonstram a associação entre o aumento das taxas de suicídio e a exposição a pesticidas (Faria et al., 2014) no Mato Grosso do Sul (Pires et al., 2005), Rio de Janeiro (Meyer et al., 2010) e Rio Grande do Sul (Meneghel et al., 2004).

1.2 Carbofurano

Um dos pesticidas mais comumente utilizados são os carbamatos, como por exemplo o carbofurano, o metomil e o tiodicarbe (Neves et al., 2020). O carbofurano ainda é utilizado em alguns países como Portugal, França e Espanha (Lewis et al., 2016), porém teve seu uso suspenso no Brasil em 2018 devido ao seu efeito tóxico na saúde humana (Pereira, 2017). A Organização Mundial da Saúde alerta para os riscos da exposição ao carbofurano, dentro eles efeitos neurotóxicos, genotóxicos, citotóxicos, mutagênicos, reprodutivos, endócrinos e dermatológicos (World Health Organization and Safety, 2010).

Apesar de descontinuado, alguns estudos mostraram a presença de carbofurano em alimentos e em águas de superfície tanto no Brasil como em outros países. Em alimentos, o carbofurano foi encontrado acima do limite máximo em pepinos (0,146 μg/kg) (Song et al., 2018), néctar e pólen da canola (35,78 μg/kg) (Wen et al., 2021) e em amendoins no Camarões (0,0966 μg/kg) (Galani et al., 2020). Também foram identificados traços de carbofurano (de 0,018 a 0,167 μg/L) em 50% das amostras de refrigerantes preparados a base de frutas coletadas mundialmente (Castilla-Fernández et al., 2021). Em rios do estado de Santa Catarina (Brasil), aproximadamente 7% das amostras apresentaram detecção de carbofurano nos anos de 2013 e 2014 (Vieira et al., 2016). Na Lagoa dos Patos foi detectado 1,40 μg/L de carbofurano (Silva et al., 2009), no Rio Piratini 11,76 μg/kg (Grützmacher et al., 2008) e uma alta concentração (148 μg/L) em rios da região centro-oeste do Brasil (Brovini et al., 2023). Isso pode ser devido ao uso indevido do pesticida e/ou ao uso de carbossulfano e de benfuracabe que produzem carbofurano como metabólito no ambiente, sendo mais persistente do que os compostos primários (Iesce et al., 2006; Trevisan et al., 2004; Zhang et al., 2016).

O carbofurano é comercializado na fórmula de Furadan®, um sólido cristalino com leve odor fenólico, altamente solúvel em água (352 mg/dL a 25°C) com baixo coeficiente de adsorção no solo (Koc = 30). Koc é, portanto, o coeficiente de partição do contaminante entre solo-água corrigido pela matéria orgânica do solo (D'Agostinho and Flues, 2006), o que exemplifica a baixa retenção do carbofurano no solo. A principal fonte de contaminação do carbofurano no meio ambiente ocorre por aplicação direta na cultura ou contaminação indireta através da sua mobilidade no solo e volatilização, o que permite contaminar fontes de água próximas, plantas e animais (PubChem; Tudi et al., 2022). A quantidade de pesticida aplicada não atinge somente os organismos-alvo já que grande parte da aplicação é dispersada, contaminando o ar, solo e ecossistemas (Mishra et al., 2020; Vryzas, 2018). A meia-vida desse composto é variável, sendo reportada em 36 dias na água e 75 dias no solo, sendo resistente à degradação (Mishra et al., 2020). O baixo coeficiente de adsorção do carbofurano favorece sua contaminação em superfícies e águas subterrâneas. O pH do solo é um fator determinante da persistência desse pesticida, já que o carbofurano tende a estabilidade em solos ácidos em relação a solos neutros ou alcalinos (Campbell et al., 2004; PubChem). O destino ambiental e a sua persistência depende de diversos fatores como pH, tipo de solo, a temperatura do ambiente, umidade do solo/planta, população bacteriana presente no solo e o tipo de formulação utilizada (Mishra et al., 2020). No solo, o carbofurano pode ser degradado em metabólitos através de degradação química ou microbiológica e na água por fotólise ou foto-oxidação (Fenoll et al., 2013; Mishra et al., 2020). A figura 1 exemplifica a rota ambiental do carbofurano, desde sua aplicação, contaminação e degradação.

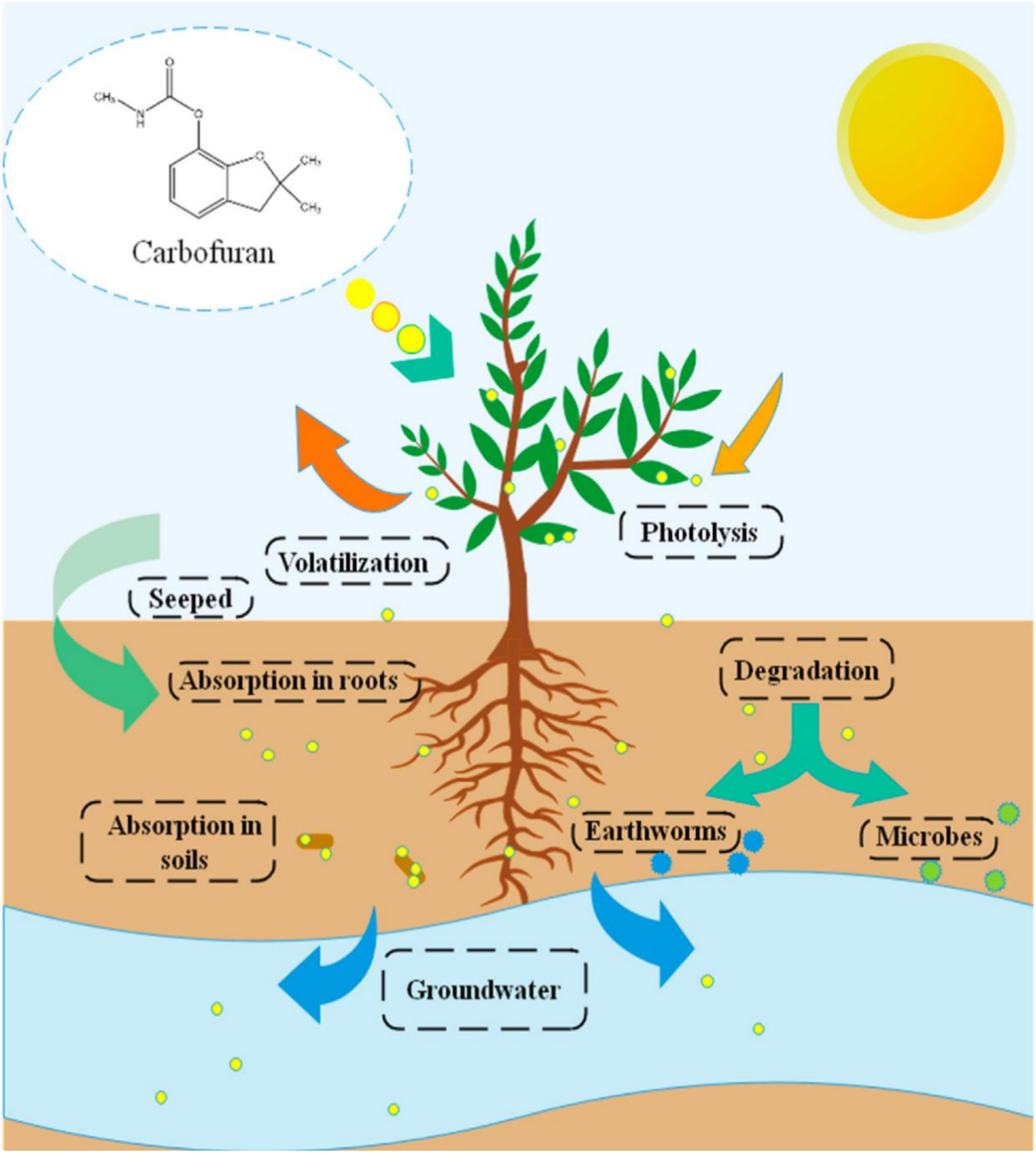

Figura 1. Destino ambiental do carbofurano (Fonte autorizada: Mishra et al., 2020).

O carbofurano pode ser absorvido pelas vias oral, respiratória e cutânea de organismos não-alvo. São inibidores reversíveis da acetilcolinesterase (AChE), causando uma carbamilação nessa enzima que degrada a acetilcolina impedindo a inativação da acetilcolina e permitindo uma ação prolongada desse neurotransmissor na fenda sináptica, levando a uma hiperexcitabilidade dos receptores colinérgicos (tanto nicotínicos como muscarínicos) e a uma síndrome colinérgica. Essa síndrome pode produzir sintomas como náuseas, vômito, fraqueza, dor e, em casos graves, coma e morte (Araújo et al., 2016)

Em estudos pré-clínicos, a exposição ao carbofurano (0,625 mg/kg) induziu alterações de hiperestimulação colinérgica em frangos (Lehel et al., 2010). Em ratos (1mg/kg), aumentou o estresse oxidativo e reduziu a atividade da acetilcolinesterase (Kamboj et al., 2008). Em ratas adultas, o carbofurano (0,4, 0,7, 1 e 1,3 mg/kd/dia) alterou o ciclo estral e afetou os folículos ovarianos, alterando a qualidade reprodutiva dos animais (Baligar and Kaliwal, 2002). Em peixes-zebra, o carbofurano (5, 50 e 500 μg/L) induziu um efeito ansiogênico e aumentou a atividade da enzima tirosina hidroxilase (Liu et al., 2020), enquanto em lambaris, esse composto (10, 50, 100 e 500 μg/L) aumentou o movimento opercular e diminuiu a velocidade de nado, aumentando o consumo de oxigênio (Mendes et al., 2021). Ainda em peixes, induziu danos oxidativos em carpas (50 μg/L) (Clasen et al., 2014) e diminuiu a velocidade de nado e inibiu a acetilcolinesterase em robalos (31, 63, 125 e 250 μg/L) (Hernández-Moreno et al., 2011).

Considerando a problemática levantada do uso disseminado de pesticidas e seus efeitos deletérios em seres humanos e outros organismos não-alvo, se faz necessário o estudo dos efeitos de concentrações ambientais do carbofurano sobre parâmetros comportamentais e neuroquímicos em peixes-zebra. De ampla homologia genética com o ser humano, o peixe-zebra é um animal que pode facilmente mimetizar as condições de um animal no ecossistema exposto ao pesticida. Porém, existem poucos estudos sobre os efeitos do carbofurano de concentrações ambientais e os seus efeitos comportamentais e neuroquímicos nessa espécie.

2. OBJETIVO GERAL

Avaliar os efeitos da exposição ao carbofurano em concentrações ambientais (5, 50 e 500 µg/L) sobre parâmetros comportamentais e neuroquímicos em peixes-zebra adultos.

2.1 Objetivos específicos

2.1.1 Avaliar o efeito do carbofurano sobre parâmetros comportamentais como distância percorrida, velocidade média, número de cruzamentos, tempo e número de entradas na zona superior e inferior do tanque, tempo e números de episódios de imobilidade no teste de tanque novo em peixes-zebra adultos;

2.1.2 Avaliar o efeito do carbofurano sobre parâmetros comportamentais como tempo e número de entradas na zona de interação, distância percorrida, velocidade média e número de cruzamentos no teste de preferência social em peixe-zebra adulto;

2.1.3 Avaliar o efeito do carbofurano sobre parâmetros neuroquímicos (níveis de tióis não-proteicos [NPSH], substâncias reativas ao ácido tiobarbitúrico [TBARS] e atividade da acetilcolinesterase [AChE]) em encéfalos de peixes-zebra adultos.

3. ARTIGO

Os resultados desse trabalho são apresentados na forma de artigo científico completo aceito para publicação na revista *Comparative Biochemistry and Physiology Part C* (doi: 10.1016/j.cbpc.2024.109969)**.**

Evaluation of behavioral and neurochemical changes induced by carbofuran in zebrafish (*Danio rerio*)

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Abstract

Carbofuran (CF) is a carbamate class pesticide, widely used in agriculture for pest control in crops. This pesticide has high toxicity in non-target organisms, and its presence in the environment poses a threat to the ecosystem. Research has revealed that this pesticide acts as an inhibitor of acetylcholinesterase (AChE), inducing an accumulation of acetylcholine in the brain. Nonetheless, our understanding of CF impact on the central nervous system remains elusive. Therefore, this study explored how CF influences behavioral and neurochemical outcomes in adult zebrafish. The animals underwent a 96-hour exposure protocol to different concentrations of CF (5, 50, and 500 µg/L) and were subjected to the novel tank (NTT) and social preference tests (SPT). Subsequently, they were euthanized, and their brains were extracted to evaluate neurochemical markers associated with oxidative stress and AChE levels. In the NTT and SPT, CF did not alter the evaluated behavioral parameters. Furthermore, CF did not affect the levels of AChE, non-protein sulfhydryl groups, and thiobarbituric acid reactive species in the zebrafish brain. Nevertheless, further investigation is required to explore the effects of environmental exposure to this compound on non-target organisms.

Keywords: Carbofuran, pesticides, behavior, *Danio rerio*, acetylcholinesterase, oxidative stress

Introduction

Pesticides are used in agricultural management, pest, and disease control to increase productivity and profit. However, the widespread use of these compounds has raised numerous questions due to their potential to affect non-target organisms in the ecosystem, including humans (Damalas and Eleftherohorinos 2011; Dutra and Ferreira 2017; Neves et al. 2020). These molecules also have various ways of contaminating non-target organisms, such as through water, food, or air (Xiong et al. 2023), which makes humans an easy target for contamination. The increase in pesticide production and its widespread use over the last decade has resulted in a significant public health problem due to exposure to these agrochemicals (Brovini et al. 2023). The extent of this contamination varies according to the physical and chemical properties of each compound (such as half-life, stability, and combination with other compounds that act as carriers) and soil characteristics (such as microbiota, pH, porosity, water concentration, leaching process, compound dissipation, and soil temperature) (Vryzas 2018; Mishra et al. 2020; Hu et al. 2022).

Carbofuran (CF) is a widely used carbamate class pesticide that has been detected in rivers worldwide. In Brazil, the detection of CF ranged from 1.40 µg/L to 148 µg/L (Grützmacher et al. 2008; Brovini et al. 2023) and, in China, from 1.54 µg/L to 204 µg/L (Zhang et al. 2016). The highest concentration found in Kenya was 495 µg/L (Otieno et al. 2010). In food, CF has been found above the maximum limit in cucumbers (0.146 µg/kg) (Song et al. 2018), nectar, and rapeseed pollen (35.78 µg/kg) (Wen et al. 2021), and in peanuts in Cameroon (0.0966 µg/kg) (Galani et al. 2020). CF presents high environmental persistence due to its physicochemical characteristics. It has a half-life of 36 days in water and 75 days in soil, making it resistant to degradation. Additionally, it is highly soluble in water (351 mg/dL), has high mobility in various soil types, and has a low adsorption coefficient (Koc=30), enhancing its contamination potential in aquatic environments (Mishra et al. 2020). It also remains stable in acidic or neutral pH soils, further contributing to its persistence in the environment (Mishra et al. 2020; Tang et al. 2021; Brovini et al. 2023). CF can be absorbed through oral, respiratory, and dermal routes in non-target organisms, causing a cholinergic syndrome, leading to symptoms such as nausea, vomiting, weakness, pain, and, in severe cases, coma and death (Mishra et al. 2020).

In zebrafish, exposure to CF increases anxiety and tyrosine hydroxylase activity (Liu et al. 2020). Furthermore, exposure to CF induced oxidative damage in carp (*Cyprinus carpio*), leading to alterations in superoxide dismutase, catalase, glutathione S-transferase levels, and thiobarbituric acid reactive species (TBARS) levels (Clasen et al. 2014). Additionally, the impacts of CF exposure on sea bass (*Dicentrarchus labrax*) included decreased swimming speed and inhibition of acetylcholinesterase (AChE) (Hernández-Moreno et al. 2011). Studies on CF exposure in humans have shown an association with the development of neurodegenerative diseases (Kamel et al. 2007; Tang et al. 2021), alterations in sperm quality, and testosterone levels (Abell et al. 2000). The literature has also explored the relationship between exposure to carbamates and mental disorders such as depression and anxiety (Freire and Koifman 2013).

Considering the widespread use of CF and its potential toxicity to non-target organisms, this study aimed to assess the effects of CF on behavioral and neurochemical parameters in adult zebrafish.

Materials and Methods

Animals

We conducted experiments using 160 adult short-fin wild-type zebrafish (*Danio rerio*, Hamilton, 1822), aged between 4 and 6 months, with an equal distribution of males and females (1:1 ratio) acquired from a local commercial supplier (Delphis, RS, Brazil). Fish were acclimated to the laboratory conditions for at least 2 weeks. The animals were housed in unenriched 16-L tanks (40×20×24 cm, maximum density of two fish per liter) and under a 14/10-h light/dark cycle (lights on at 7 a.m. and off at 9 p.m.). The water in the tanks adhered to controlled conditions necessary for zebrafish (see details in Benvenutti et al. 2021). Aeration and filtration of water were consistently maintained using mechanical, biological, and chemical systems. The fish were fed twice daily with a combination of commercial flake food (Poytara®, Brazil) and brine shrimp (*Artemia salina*). The animals were euthanized by hypothermic shock followed by decapitation after the completion of behavioral testing, according to the AVMA Guidelines for the Euthanasia of Animals (Leary et al. 2020). The animals were subjected to chilled water with temperatures ranging from 2 to 4 °C for at least 5 minutes following the loss of posture and cessation of opercular movements. Afterward, decapitation was performed as a secondary measure to ensure death. Following euthanasia, the sex of each animal was confirmed by dissection of gonadal tissue. Since no sex-specific effects were observed in any of the experiments, the data were combined for analysis. The animal welfare and ethical review committee at the Universidade Federal do Rio Grande do Sul (UFRGS) approved all procedures conducted in this study (approval #42114/2022).

Chemicals

CF was obtained from Sigma-Aldrich™ (CAS 1563-66-2) (St. Louis, MO, USA). Biochemical assay reagents, including bovine serum albumin (CAS Number: 9048-46-8), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (CAS Number: 69-78-3), thiobarbituric acid (TBA) (CAS Number: 504-17-6), and trichloroacetic acid (TCA) (CAS Number: 76-03-9), were obtained from Sigma Aldrich (St. Louis, MO, USA). Additionally, absolute ethanol (CAS Number: 64-17-5) was acquired from Merck KGaA (Darmstadt, Germany).

Experimental design

The animals were allocated to the following experimental groups: control (CTRL, n=40) and CF (5, 50, and 500 µg/L, n=40). The concentrations were determined using environmental concentrations that had been previously reported (Otieno et al. 2010; Zhang et al. 2016; Liu et al. 2020; Brovini et al. 2023). Allocation to experimental groups followed block randomization procedures to counterbalance sex and two different housing tanks. The animals of the different experimental groups were allocated in the same room under the same conditions of temperature, luminosity, and vertical level. The experiments were carried out in two batches, with half the sample size in each batch.

During the drug exposure period, the fish were housed in 4-L static tanks (18×18×18 cm), with two tanks being employed within each concentration to reduce the tank-related effects and remained there for 96 consecutive hours (OECD 203, 2019). Tanks were coded to keep caregivers blinded to the treatment group and barriers were set to visually isolate the animals from different groups. CF was dissolved in ultrapure water to create a stock solution of 40 mg/mL, which was then utilized to prepare diluted solutions for the exposure. The final test concentrations were achieved by adding specific amounts of the stock solution to the water in the home tanks.

After 96 h of exposure, a set of animals was individually submitted to the novel tank test (NTT) (n=24), and a different set of animals to the social preference test (SPT) (n=16). The order of outcome assessment followed block randomization procedures to counterbalance for treatment groups. Animal behavior was recorded (Logitech® C920 HD webcam) and analyzed by researchers who were blinded to the experimental conditions. The analysis was conducted using ANY-Maze™ tracking software (Stoelting Co., based in Wood Dale, IL, USA). All tests were conducted between 08:00 a.m. and 12:00 p.m. (during the light phase). After the completion of the tests, the animals were euthanized, and their brains were promptly dissected and homogenized for the neurochemical assays. The brains of the animals submitted to NTT were prepared for analyses of oxidative stress, with a pool of 4 brains per sample. After the SPT, the animals were euthanized, and the brains of these animals were collected and used to analyze the AChE activity. The neurochemical parameter analyses were as follows: non-protein sulfhydryl groups (NPSH) (n=6), TBARS (n=6), and acetylcholinesterase (AChE) activity (n=16). Figure 1 illustrates the experimental design.

We report details of our sample size estimation, all manipulations, and measures employed in the study. Raw data have been deposited in the Open Science Framework and are openly accessible at osf.io/2dez8/ (Rolim et al. 2023).

Novel Tank Test (NTT)

The NTT was conducted by placing animals individually in the apparatus (24×8×20 cm) filled with water at optimal conditions (15 cm water level) and recording their behavior for 6 minutes, (Marcon et al. 2018; Mocelin et al. 2019; Bertelli et al. 2021). To prevent any potential interference from drug residues or alarm substances released by previously tested fish, the water in the tanks was changed between each animal. The behavior of the animals was assessed using the ANY-maze™ software, with which we virtually divided the tank into three equal horizontal zones (upper, middle, and bottom). Quantified parameters were total distance traveled, mean speed, line crossings between tank zones, time spent, and the number of entries in the upper and bottom zones of the tank.

Social Preference Test (SPT)

The SPT was conducted following previously established protocols (Benvenutti et al. 2021; Giongo et al. 2023). In the SPT, individual fish were placed in a central tank (30×10×15cm), with two identical tanks (15×10×13 cm) on each side. They were then filmed from a frontal view for 7 minutes. On one side of the central tank, there was a tank containing only water, serving as a neutral stimulus, while the tank on the other side contained 10 zebrafish, providing a social stimulus. All tanks were filled with water at a consistent level of 10 cm and maintained under the same conditions. To prevent potential biases, the side of the tank containing the social stimulus was counterbalanced between trials. The water in the test tanks was changed between animals to prevent any interference from drug residues or alarm substances released by previously tested fish. The analyses were carried out with ANY-Maze™ software with the test tank virtually divided into three equal zones (interaction, middle, and neutral). The interaction zone was positioned adjacent to the tank containing the social stimulus, while the neutral zone was adjacent to the neutral stimulus. At the beginning of the test, animals were placed in the middle zone and given 2 minutes to acclimate to the test tank. Subsequently, their behavior was recorded and analyzed for 5 minutes. The parameters quantified were time and entries in the interaction zone, distance traveled, mean speed, and the number of crossings between tank zones.

Biochemical Parameters

Following the behavioral tests, brain samples were collected according to previously published protocols (Sachett et al. 2020c). Each independent sample involved the collection of four brains immediately following euthanasia. These brains were then pooled together and homogenized in 600 µL of phosphate-buffered saline (PBS). The homogenized mixture was centrifuged at 3000 g at 4°C using a cooling centrifuge, and the resulting supernatant was collected, which was kept in microtubes frozen at -80°C until the assays were performed (average of 2 months of storage before the analyses). The protein content was quantified according to established protocols (Sachett et al. 2020a). The following biochemical parameters were evaluated: non-protein sulfhydryl group (NPSH) levels (Sachett et al. 2021), thiobarbituric acid reactive species (TBARS) (Sachett et al., 2020b), and acetylcholinesterase (AChE) activity (Ellman et al. 1961). Methodological details can also be consulted in protocols.io (protocols.io/lapcom).

Statistical Analysis

The sample size was determined to detect effect sizes of 0.5 and 0.4, respectively, for the NTT and SPT, with a power of 0.9 and an alpha of 0.05 using G*Power 3.1.9.7 for Windows. This resulted in a total sample size of 160, equivalent to n=24 animals per experimental group in NTT and n=16 animals per experimental group in SPT. In the NTT, the primary outcome was defined as the total distance traveled by the animals. On the other hand, in the SPT, the primary outcome was defined as the time spent by the animals in the interaction zone. The normality and homogeneity of variances were assessed for all datasets using the D'Agostino-Pearson and Levene tests, respectively. Subsequently, the data were analyzed using one-way ANOVA. Post hoc analyses were conducted using Tukey's test when appropriate. All statistical analyses and graphical representations were performed using GraphPad Prism version 8.0.1 for Windows. No outliers were excluded from the behavioral analysis data. Two data points were excluded from the control group, one from the CF 5 µg/L group, two from the CF 50 µg/L group, and one from the CF 500 µg/L group in the AChE analysis due to discrepant results, likely arising from analytical errors. Data are expressed as mean ± standard deviation of the mean (SD). The level of significance was set at $p<0.05$.

Results

Behavioral Parameters

Figure 2 summarizes the acute effects of CF on adult zebrafish behavior in the NTT. At the tested concentrations, CF exposure did not induce alterations in the distance traveled ($F_{3,92}$=0.005784, p=0.9994; figure 2A), line crossings ($F_{3,92}$=0.4002, p=0.7531; figure 2B), and time ($F_{3,92}$=0.4235, p=0.7366; figure 2C) and entries in the upper zone ($F_{3,92}$=0.5735, p=0.6339; figure 2D). Exposure to CF also did not elicit changes in the mean swimming speed (Suppl. figure 1A), time (Suppl. figure 1B), and entries to the bottom zone (Suppl. figure 1C).

The acute effects of CF on adult zebrafish in the SPT are shown in figure 3. CF, in the tested concentrations, did not alter the time spent in the interaction zone ($F_{3,60}$=0.8752, p=0.4591; figure 3A), entries to the interaction zone ($F_{3,60}$=1.315, p=0.2780; figure 3B), and distance traveled ($F_{3,60}$=2.320, p=0.0844; figure 3C). Changes in the mean swimming speed (Suppl. figure 2A) and line crossings (Suppl. figure 2B) were also not observed. Table 1 summarizes the one-way ANOVA analysis results.

Biochemical Parameters

The effects of acute CF exposure on oxidative status parameters and AChE activity in zebrafish are presented in figure 4. The exposure did not induce alterations to the levels of NPSH ($F_{3,20}$=0.9166, p=0.4507; figure 4A) and TBARS ($F_{3,20}$=0.4635, p=0.7109; figure 4B), and to the activity of AChE ($F_{3,54}$=0.5667, p=0.6393; figure 4C). Table 2 summarizes the one-way ANOVA analysis results.

Discussion

CF at concentrations of 5, 50, and 500 μg/L showed no impact on zebrafish behavior during both the novel tank and social preference tests. Moreover, there were no alterations observed in the analyzed neurochemical parameters, such as NPSH, TBARS, and AChE activity.

The NTT is a well-established tool for assessing the effects of anxiety-like behavior-modulating interventions in zebrafish (Kysil et al. 2017; Johnson et al. 2023). This test examines the natural behavior exhibited when these fish are introduced to a novel environment. Zebrafish typically display an innate preference for the bottom zone, but as they acclimate, this behavior gradually shifts, leading them to explore other zones of the tank (Cachat et al. 2010). Anxiogenic interventions tend to exacerbate the zebrafish's initial preference for the bottom region, exhibiting heightened anxiety-like behavior (Kysil et al. 2017; Reis et al. 2020).

Conversely, anxiolytic treatments or interventions disrupting their natural defensive behavior encourage fish to spend more time in the upper zone (Gebauer et al. 2011; Kysil et al. 2017). Additionally, total distance traveled serves as a metric for overall locomotor activity, offering valuable insights into the hyperstimulating or sedating effects of various interventions (Cachat et al. 2010; Kysil et al. 2017). The complexity of zebrafish locomotor behavior is governed by multiple brainstem neurons and neuromodulatory pathways, whose integrity is crucial for normal functioning (Drapeau et al. 2002; Dreosti et al. 2015; Sachett et al. 2022). The behavioral parameters assessed in the NTT are vital for essential functions such as reproduction, predator avoidance, and foraging (Zhang et al. 2016). For instance, a study assessing the impact of exposure to the herbicide 2,4-D on zebrafish has observed alterations in both the time spent in the upper zone and the distance traveled, disrupting their natural behavior (Thiel et al. 2020). Furthermore, a recent meta-analysis showed that fungicides decrease the locomotion in both zebrafish larvae and adults (Reis et al. 2023). In our study, there is no evidence that CF alters any locomotor parameter.

The SPT is widely used for investigating the effects of different interventions on zebrafish, given their innate schooling behavior and intricate social dynamics, including hierarchical and breeding relationships (Dreosti et al. 2015). Being a species with complex social interactions, zebrafish engage in social behaviors as a means of escaping predation and to facilitate reproduction (Dreosti et al. 2015). Given the critical role of social preference in this species, alterations in this behavior are highly relevant, making the SPT an ideal assay for studying interventions that may disrupt the natural behavior of zebrafish. Despite findings from previous studies indicating alterations in social behavior due to exposure to different pesticides (Yan et al. 2023; Liu et al. 2023), our study did not observe any changes following exposure to CF. Since social behavior is genetically preserved and has an ontogenic nature (Buske and Gerlai 2011; Scerbina et al. 2012), it is less vulnerable to low-impact modulations, such as the concentrations used in this study, where the highest concentration corresponds to 6% of the LC_{50} of similar species, such as *Carassius auratus*, the goldfish (Dreosti et al. 2015; Valadas et al. 2023).

Reactive oxygen species are constantly produced during normal physiological events and are important in the brain signaling processes. High concentrations of oxidants can harm cell components ultimately resulting in physiological impairment and the disruption of normal cell functions. Neuronal membranes are rich in polyunsaturated fatty acids, which make the brain more vulnerable to lipid oxidation and to the damage caused by lipid peroxidation (Fedoce et al. 2018; Halliwell 2022). Within this study, there were no changes in the presented markers of oxidative stress and cellular damage. However, different oxidative stress markers of interest have been shown to be impacted by CF exposure, such as SOD and CAT enzymes (Fedoce et al. 2018; Liu et al. 2020). Another crucial aspect to consider when discussing the results found here with those already reported in the literature is the animal experimentation protocol. This study followed OECD 203 (OECD 203, 2019), a guideline that regulates and standardizes toxicological exposure studies on zebrafish. This guideline advocates exposing the animals for 96 hours before commencing behavioral tests, avoiding the use of solvents in compound dilution, and avoiding any disturbance that may alter the animal's behavior. Several studies presented here did not follow these guidelines, such as the study conducted by Liu and collaborators (Liu et al. 2020), which exposed the animals for 48 hours and handled the animals to replace the aquarium water, which is a stressful factor for the animals and could be associated with some of the behavioral changes reported, as well as the studies by other research groups that also did not follow this standardized protocol (Hernández-Moreno et al. 2011; Clasen et al. 2014; Cui et al. 2019; Mendes et al. 2021).

Carbamates, including CF, act as reversible inhibitors of AChE, causing carbamylation of this enzyme. This carbamylation prevents the degradation of acetylcholine, allowing for its prolonged action in the synaptic cleft. Consequently, it leads to hyperexcitability of both nicotinic and muscarinic cholinergic receptors, resulting in cholinergic syndrome (Gupta 1994; Mishra et al. 2020). CF has consistently been shown to inhibit AChE in different fish species (Dembélé et al. 2000; Hernández-Moreno et al. 2011). Here, on the other hand, no alteration in the activity of AChE was measured after exposure to CF. This could be attributed to the enzymatic reactivation of AChE. The zebrafish's brain regeneration after injury is complex and has several repair mechanisms, as previously reported, which could be associated with the adaptation to the damage caused by CF after AChE reactivation and normalization of enzymatic activity (Ghosh and Hui 2016).

An important aspect to be discussed is the association of CF with other compounds. CF has been detected in various samples in combination with chlorpyrifos, 2,4-D, glyphosate, and other pesticides of great toxicological importance (Buch et al. 2013; Brovini et al. 2023). While our study focused on the effects of pure CF, it is noteworthy that CF can be found in commercial products alongside other substances, such as absorption promoters, which could influence its toxicological effects in real-world scenarios. The presence of absorption promoters and interactions between CF and other pesticides or pollutants may enhance CF uptake in aquatic organisms, potentially intensifying its toxic effects compared to exposures to pure CF. A study reports the interaction between CF and carbon nanotubes, where the association of these two pollutants increases histological damage in Nile tilapia (*Oreochromis niloticus*) exposed to both compounds by 25% compared to animals exposed only to CF (Campos-Garcia et al. 2016). CF exposure in association with carbon nanotubes also significantly affected the metabolic routes of shrimp (*Palaemon pandaliformis*) (Alves et al. 2022). Sinergic effects have been observed with the association between CF and cadmium (Wang et al. 2022). Although these compounds may exhibit either absence or low toxicity when isolated, synergism can occur in the environment leading to toxicity to non-target organisms. Therefore, further studies are needed to assess the potential synergistic effect of CF and other pesticides.

Conclusion

Although the results presented in this study do not show behavioral and biochemical changes, it is still necessary to study the effects of CF under conditions closer to the real environment where the pesticide is present and causing harm to non-target organisms. While our study contributes valuable insights, further research specifically addressing behavioral and neurochemical outcomes is needed to deepen our understanding of the effects of CF on adult zebrafish. To further contribute to the fields of toxicology and the environment, it is essential to study a broader range of concentrations of this compound and the alterations it induces in different outcomes. Additionally, studying longer exposure times to environmental concentrations and their transgenerational and long-term effects at different stages of zebrafish is crucial.

Author Contributions

Conceptualization: G.R.O., M.G.-L., and A.P. **Data curation:** G.R.O. and M.G.-L. **Formal analysis:** G.R.O., M.G.-L., and A.P. **Funding acquisition:** A.P. **Investigation:** G.R.O., M.G.-L., R.C., L.M.B., S.M.P., T.S.-B., and D.G. **Methodology:** G.R.O., M.G.-L., R.C., D.G., A.T.W., A.P.H., and A.P. **Project administration:** G.R.O., M.G.-L., and A.P. **Resources:** A.T.W. and A.P. **Supervision:** A.P. **Validation:** G.R.O., M.G.-L., and A.P. **Visualization:** G.R.O., M.G.-L., and A.P. **Writing - original draft:** G.R.O. and M.G.-L. **Writing - review & editing:** R.C., L.M.B., S.M.P., T.S.-B., D.G., R.G., A.T.W., A.P.H., and A.P.

Interest Statement

The authors declare no conflicts of interest.

Data Availability

All data are available in the Open Science Framework (osf.io/2dez8/).

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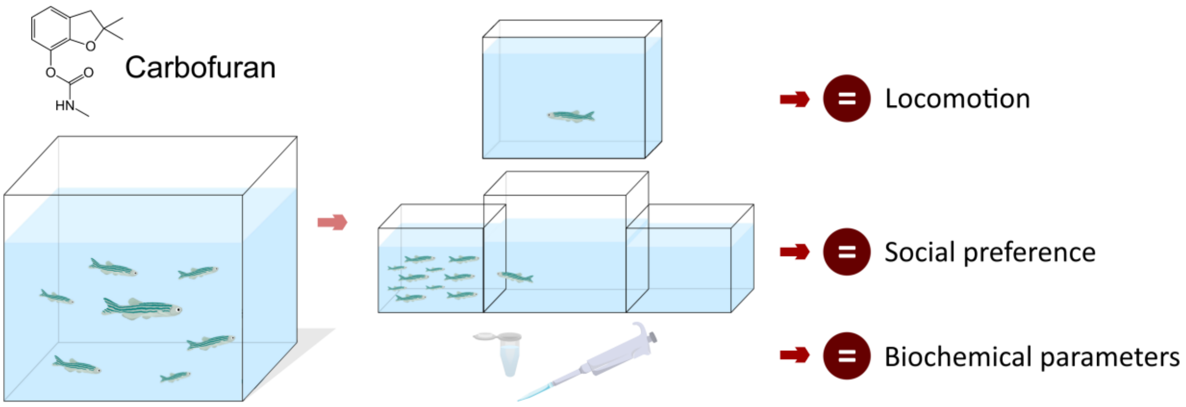
Figures



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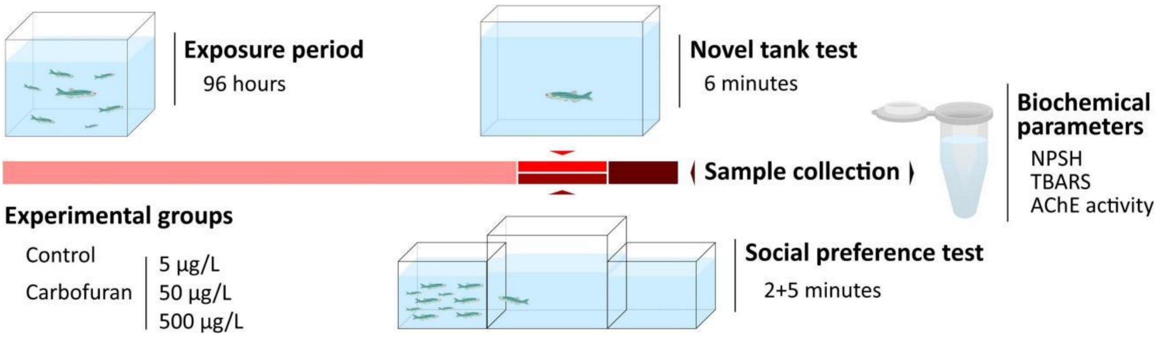

Figure 1. Overview of the experimental design evaluating the effects of acute carbofuran exposure to zebrafish. NPSH = Non-protein sulfhydryl groups; TBARS = Thiobarbituric acid reactive species; AChE = Acetylcholinesterase.

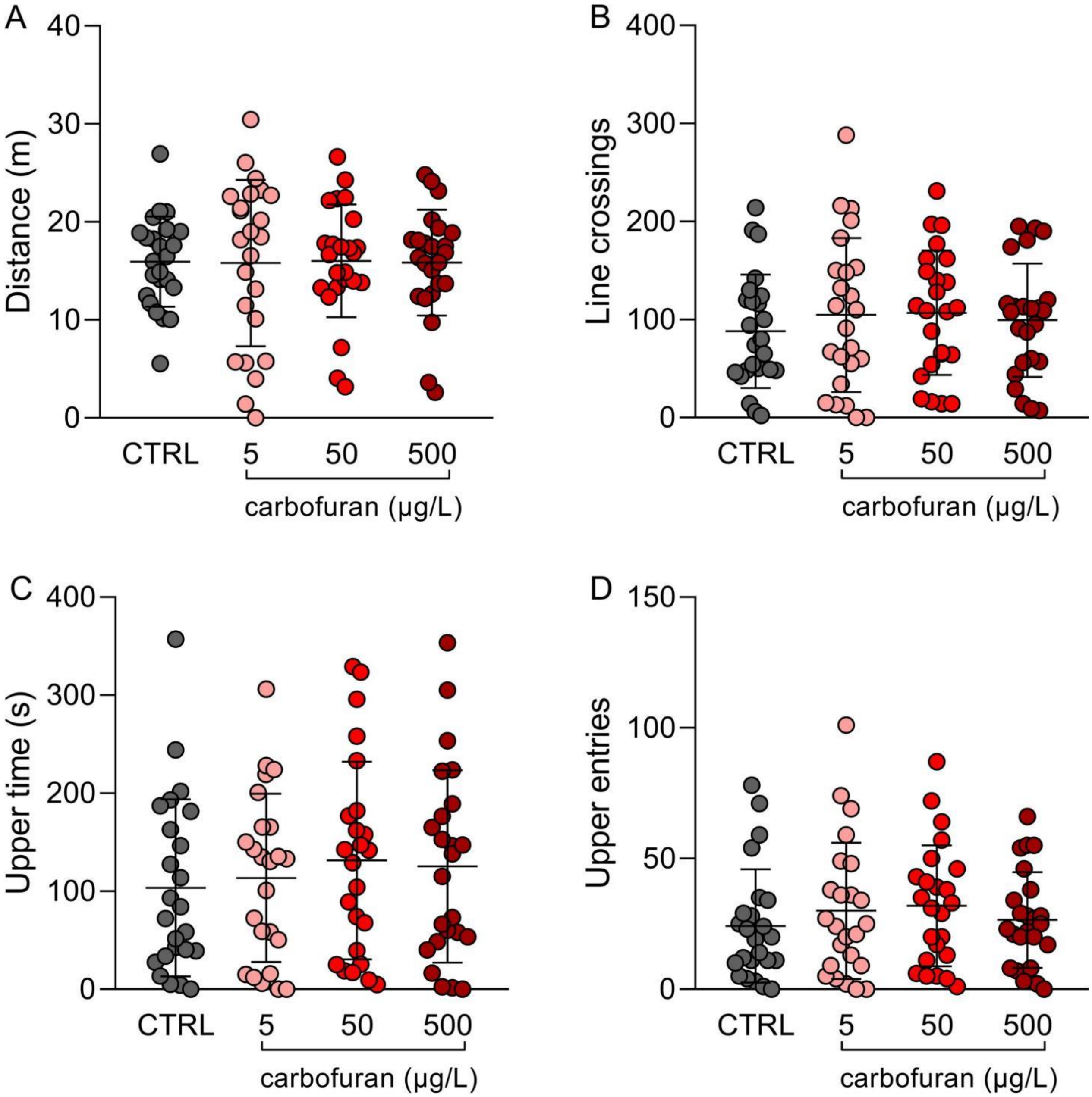

Figure 2. Effects of carbofuran (5, 50, and 500 µg/L) on behavioral parameters in the Novel Tank Test. (A) Distance, (B) Line crossings, (C) Upper time, (D) Upper entries. Data are expressed as mean ± S.D. One-way ANOVA. n=24. CTRL = Control.

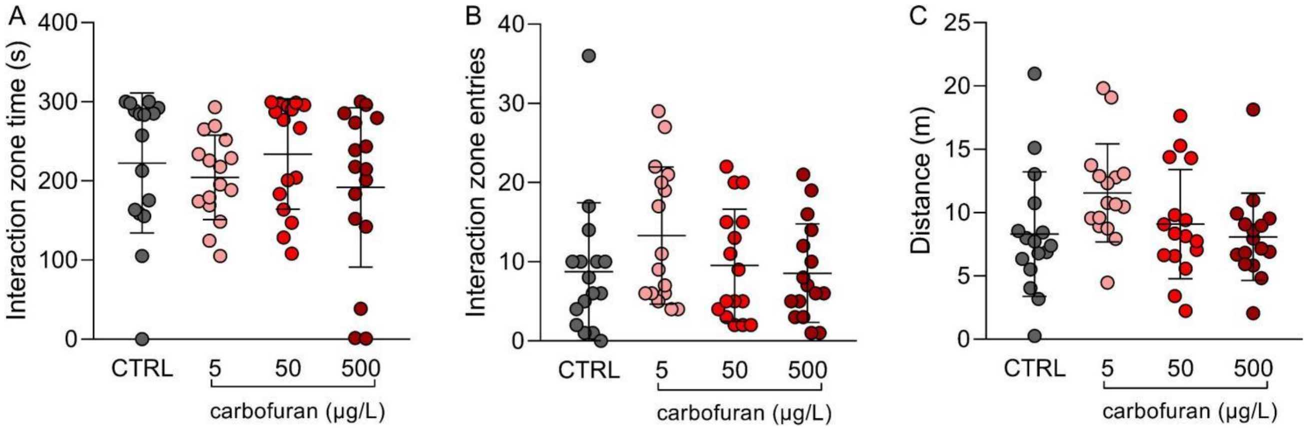

Figure 3. Effects of carbofuran (5, 50, and 500 µg/L) on behavioral parameters in the Social Preference Test. (A) Interaction time, (B) Interaction entries, and (C) Distance. Data are expressed as mean ± S.D. One-way ANOVA. n=16. CTRL = Control.

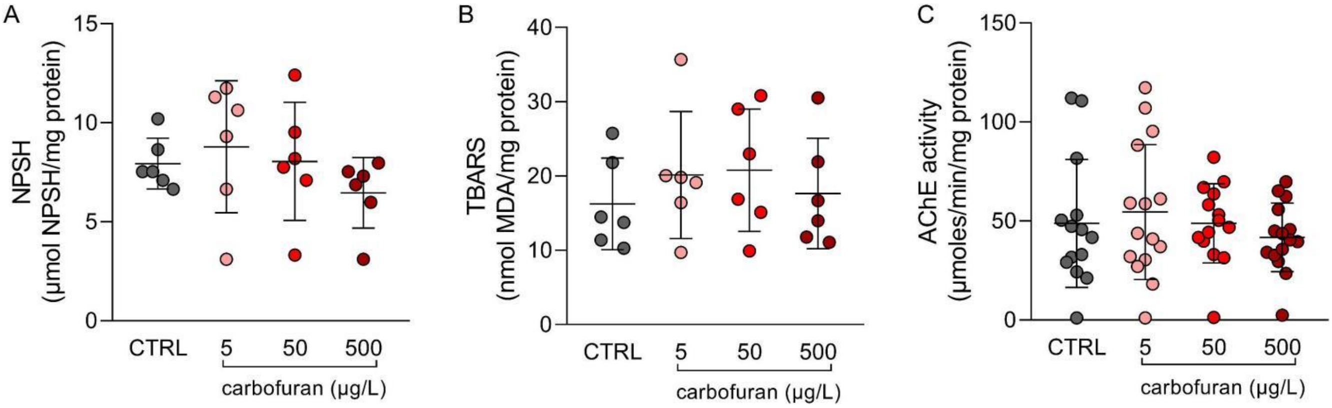

Figure 4. Effects of carbofuran (5, 50, and 500 µg/L) on neurochemical parameters. (A) NPSH (Non-protein sulfhydryl groups), (B) TBARS (Thiobarbituric acid reactive species), (C) AChE (Acetylcholinesterase) activity. Data are expressed as mean ± S.D. One-way ANOVA. n=6 for NPSH and TBARS, n=14-15 for AChE (n=14 CTRL; n=15 CF5; n=14 CF50; n=15 CF500). CTRL = Control.

Tables

Table 1. Summary of the one-way ANOVAs for the NTT and SPT.

Test	Dependent Variable	F-Value	DF	p-value
NTT	Distance	0.005784	3, 92	0.9994
	Line crossings	0.4002	3, 92	0.7531
	Upper time	0.4235	3, 92	0.7366
	Upper entries	0.5735	3, 92	0.6339
	Time in the interaction zone	0.8752	3, 60	0.4591
SPT	Interaction zone entries	1.315	3, 60	0.2780
	Distance	2.320	3, 60	0.0844

DF = Degrees of freedom; NTT = Novel Tank Test, SPT = Social Preference Test

Table 2. Summary of the one-way ANOVAs for the neurochemical analyses.

Test	F-Value	DF	p-value
NSPH	0.9166	3, 20	0.4507
TBARS	0.4635	3, 20	0.7109
AChE activity	0.5667	3, 54	0.6393

DF = Degrees of freedom; NPSH = Non-protein sulfhydryl groups; TBARS = Thiobarbituric acid reactive species; AChE = Acetylcholinesterase.

4. DISCUSSÃO

Esse estudo avaliou os efeitos do carbofurano em concentrações ambientais em peixes-zebra machos e fêmeas adultos. Nas concentrações estudadas, carbofurano não alterou o comportamento dos peixes-zebra nos testes de tanque novo e comportamento social. Nas análises neuroquímicas, o carbofurano não induziu efeitos nos parâmetros analisados.

O teste de tanque novo é amplamente utilizado para avaliar parâmetros locomotores e ansiedade em peixes-zebra (Marcon et al., 2016; Mocelin et al., 2019b; Yoshida, 2022). Nesse teste, o animal tende a explorar porções inferiores (fundo) do aquário inicialmente e, com o passar do tempo, explorar zonas superiores, ou seja, mais próximas da superfície. Intervenções que reduzem a ansiedade causam um aumento na exploração da zona superior do aquário, enquanto intervenções ansiogênicas causam comportamento oposto (Anwer et al, 2021; Bertelli et al., 2021; Marcon et al., 2018). Estudos mostraram que a exposição ao carbofurano (5, 50 e 500 μg/L) por 48 horas diminuiu o tempo de permanência no topo do aquário bem como o número de entradas, além de aumentar o número de movimentos erráticos em peixes-zebra adultos (Liu et al., 2020). Já a exposição em larvas de 3 horas pós fertilização por 96 horas a concentrações subletais (0,01, 0,1 e 1 mg/L) de carbaril (outro composto carbamato) não alterou o padrão de nado no teste de tanque novo quando adultos (Correia et al., 2019). Em uma exposição de 96 horas à concentração de 165 mg/L de carbofurano em gambusias (peixes de água doce) houve uma redução na velocidade e na distância percorrida (Nunes, 2020).

O comportamento social é importante e garante a coesão do cardume, auxilia na busca por alimento e por parceiros para reprodução e a confere maior segurança aos peixes-zebra. O teste de preferência social é uma ferramenta que auxilia no estudo de endofenótipos relacionados a doenças neuropsiquiátricas e pode ser modulado pela exposição a fármacos, drogas, pesticidas e hormônios (Benvenutti et al., 2020; Do Nascimento and Maximino, 2023). No nosso trabalho, não foram observados efeitos significativos no teste de preferência social. Esse achado pode estar atrelado ao fato de que o comportamento social do animal é complexo e dependente de diversos fatores, como pistas visuais, olfatórias e de substâncias de alerta liberada pelos seus coespecíficos (Engeszer et al., 2007; Engeszer et al., 2004; Valadas et al., 2022). A concentração usada aqui foi cerca de 6% da DL50 de espécies similares ao peixe-zebra, o que pode ser insuficiente para alterar o comportamento em 96 horas de exposição (Dreosti et al., 2015; Valadas et al., 2022).

O dano por espécies reativas de oxigênio causada por agentes exógenos normalmente é prevenido por sistemas antioxidantes (Fedoce et al., 2018; Halliwell, 2022; Severo et al, 2020).

Estudos demonstram a ação de carbofurano no estado oxidativo, com aumento de GST (glutationa S-transferase, enzima envolvida no processo de biotransformação de xenobióticos) em larvas de peixe-zebra expostas a 96 horas na água coletada do rio Vacacaí, em Santa Maria (Brasil) (Severo et al, 2020), com a diminuição de defesas enzimáticas como SOD (superóxido dismutase), CAT (catalase) e GST no fígado de ratos que foram expostos durante 28 dias a 1,5 mg/kg de carbofurano (Mondal et al., 2021). Em peixes-zebra adultos expostos a 0,0097 mg/L de carbofurano por 96 horas, houve o aumento da SOD hepática (Wang et al., 2022). Um estudo realizado em encéfalos de peixe-zebras expostos a carbofurano (10 e 100 μg/L) durante 90 dias mostrou aumento nos níveis de GSH e diminuição da atividade da AChE (Hernández-Moreno et al., 2011).

A acetilcolinesterase (AChE) é uma enzima importante para o adequado funcionamento das sinapses colinérgicas presentes no sistema nervoso central e periférico. Estudos demonstram a diminuição dessa enzima no encéfalo de carpas após exposição por 96 horas de carbofurano (1 mg/L), além da diminuição da atividade enzimática com exposição a outros pesticidas, o diazinon (1,9 mg/L) e clorpifirós (3,6 mg/L) (Dembélé et al., 2000). Um estudo realizado em carpas que foram expostas a 50 ug/L da formulação comercial de carbofurano durante 30 dias, mostrou a diminuição nos níves de AChE quando analisadas no cérebro e músculo (Clasen et al, 2014). Tencas expostas a três diferentes níveis de carbofurano (0, 10 e 100 ug/L) durante 90 dias exibiu uma diminuição da atividade da AChE (Hernandez-Moreno et al, 2010). Este mesmo estudo demonstrou através de um ensaio de reativação da AChE, onde os valores demonstraram-se maiores que os controles após a reativação, indicando que possa existir uma reativação ou superprodução de AChE, como alguns autores sugerem, como sistema de proteção para neutralizar o efeito tóxico.

Esse trabalho apresenta algumas limitações que serão descritas a seguir. Uma limitação do presente estudo foi o uso do composto químico isolado, ao invés do uso de fórmulas comerciais que são normalmente utilizadas na agricultura e as quais os animais estão expostos. Carbofurano apresenta diferença de toxicidade quando comparado a sua formulação comercial, o Furadan®. Estudos realizados em *Paramecium caudatum* (um protozoário ciliado) demonstram que a formulação comercial foi mais tóxica do que o ingrediente ativo, causando aumento da mortalidade (Mansano et al., 2016) e diminuição da taxa de crescimento corporal e reprodução dos protozoários (Mansano et al., 2020). Em associação com nanotubos de carbono, o carbofurano aumentou em 25% o dano histológico nas guelras de tilápias expostas a ambos os compostos em comparação com a exposição única ao pesticida. Estes danos histológicos incluíram o deslocamento de células epiteliais, hiperplasia celular, aneurisma, dilatação e desalinhamento dos capilares. Todos os grupos demonstraram uma relação dose-dependente com as alterações histológicas (Campos-Garcia et al., 2016). Já o carbofurano combinado com cádmio induziu efeitos agudos sinérgicos, aumentando marcadores de estresse oxidativo (SOD e CAT), efeitos endócrinos (aumento de vitelogenina e tiroxina no fígado de peixes-zebra fêmeas) e aumento do apoptose celular em peixes-zebra adultos e em estágios larvais (Wang et al., 2022). Outra limitação refere-se ao tempo de exposição para a indução de alterações nos parâmetros avaliados. Dessa forma, mais estudos são necessários para determinar os efeitos das preparações comerciais contendo esse pesticida bem como a exposição em longo prazo nessa espécie.

5. CONCLUSÃO

O carbofurano, nas condições testadas aqui, não alterou o comportamento dos animais nos testes de tanque novo e de preferência social. Além disso, não houve alterações significativas nos níveis de tióis não-proteicos, substâncias reativas ao ácido tiobarbitúrico e atividade da acetilcolinesterase em encéfalos de peixes-zebra adultos. Mais estudos são necessários para determinar o impacto de preparações comerciais contendo esse pesticida, bem como a exposição a longo prazo sobre os parâmetros avaliados nesse trabalho.

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7. ANEXOS

7.1 CARTA DE APROVAÇÃO DA CEUA - UFRGS

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PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 42114
Título: Avaliação de alterações comportamentais e neuroquímicas induzidas pelo carbofurano em peixes-zebra (Danio rerio)

Vigência: 15/03/2022 à 08/09/2023

Pesquisadores:

Equipe UFRGS:

ÂNGELO LUIS STAPASSOLI PIATO - coordenador desde 15/03/2022
GIOVANA ROLIM DE OLIVEIRA - desde 15/03/2022
Carlos Guilherme Rosa Reis - desde 15/03/2022
Rafael Chitolina - desde 15/03/2022
ROSANE GOMEZ - pesquisador desde 15/03/2022
Ana Paula Herrmann - pesquisador desde 15/03/2022

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metodológicos, para a utilização de 576 peixes-zebra adultos, que serão provenientes do Departamento de Bioquímica da UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Segunda-Feira, 16 de Maio de 2022

MAITE DE MORAES VIEIRA
Coordenador da comissão de ética

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