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O impacto dos agrotóxicos nos peixes: parâmetros iniciais e efeitos persistente e transgeracional.

TESE DE DOUTORADO

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Passo Fundo, RS, Brasil
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O impacto dos agrotóxicos nos peixes: parâmetros iniciais e efeitos persistente e transgeracional.

Aline Pompermaier

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"O IMPACTO DOS AGROTÓXICOS NOS PEIXES: PARÂMETROS INICIAIS E EFEITOS PERSISTENTE E TRANSGERACIONAL"

Elaborada por
Aline Pompermaier

Como requisito parcial para a obtenção do grau de
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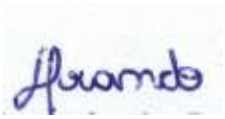

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2,4-D: Ácido diclorofenóxiacético

ACh: Acetilcolina

AChE: Acetilcolinesterase

CAS Number: Número único de registro dos compostos químicos no banco de dados do *Chemical Abstracts Service.*

CEUA: Comissão de Ética no Uso de Animais

Cm: centímetro

CONCEA: Conselho Nacional de Controle de Experimentação Animal

CTL/CAT: Catalase

dpf: Dias pós-fertilização

DNA: Ácido Desoxirribonucleico

g/L: grama por litro

h: Horas

hpf: Horas pós-fertilização

nM: Nanômetro

Min: Minuto

mL: Mililitro

pH: Potencial de hidrogênio

SisGen: Sistema Nacional de Patrimônio Genético e do Conhecimento Tradicional Associado

SOD: Superóxido dismutase

ug/L: Micrograma por litro

UPF: Universidade de Passo Fundo

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

O IMPACTO DOS AGROTÓXICOS NOS PEIXES: PARÂMETROS INICIAIS E EFEITOS PERSISTENTE E TRANSGERACIONAL.

Autora: Aline Pompermaier
Orientador: Prof. Dr. Leonardo José Gil Barcellos
Passo Fundo, 31 de Julho de 2022.

A presença de resíduos de poluentes emergentes, como agrotóxicos, é uma realidade em todo o mundo. Provenientes de diferentes fontes, como aplicação direta, lixiviação das lavouras ou processos naturais de degradação seus efeitos em espécies não-alvo ainda são pouco conhecidos. O peixe-zebra é um excelente modelo para estudos ecotoxicológicos, pois apresenta alta prolificidade, embriões translúcidos, fecundação externa e rápido desenvolvimento. Os efeitos da contaminação dos peixes por concentrações ambientais foram avaliados pela exposição dos embriões durante o período de organogênese aos herbicidas mais utilizados atualmente, glifosato (GBH) e 2,4-D (DBH). Usamos concentrações ambientais de 4,8 ug / L para glifosato e 3,4 ug / L para 2,4-D. Em nosso primeiro artigo, avaliamos os parâmetros iniciais de desenvolvimento (mortalidade, eclosão, movimento espontâneo e frequência cardíaca), parâmetros comportamentais (teste de campo aberto e estímulo aversivo) e também os possíveis mecanismos de ação (SOD, CAT e AChE). Em nosso segundo artigo, avaliamos os efeitos persistentes da exposição nos animais adultos através dos testes do tanque novo e aversidade e depois realizamos a reprodução desses animais para verificar os efeitos transgeracionais na geração F1, com as mesmas análises da F0 (desenvolvimento inicial, parâmetros comportamentais e biomarcadores bioquímicos). Na geração F0, a exposição ao glifosato diminuiu a sobrevivência, causou hipermobilidade e comportamento ansiolítico, afetou negativamente o comportamento anti-predatório das larvas e aumentou a atividade da acetilcolinesterase, enquanto a exposição ao 2,4-D causou apenas ligeira hipermobilidade nas larvas e aumento da atividade da acetilcolinesterase. Na fase adulta, os peixes expostos ao GBH apresentaram hipermobilidade e sua reação antipredatória foi prejudicada, caracterizando um efeito persistente. As larvas da F1, dos peixes expostos ao GBH, tiveram alterações comportamentais e de sobrevivência, bem como efeitos na atividade da AChE e enzimas antioxidantes, caracterizando um efeito transgeracional. Quanto ao DBH, os peixes não apresentaram persistência dos efeitos na fase adulta, porém, não foram capazes de se reproduzir, o que demonstra que a exposição afetou a perpetuação da espécie. Essas alterações observadas em todas as fases dos animais podem comprometer a perpetuação da espécie, a busca por parceiros/alimentos e facilitar a ação de predadores, o que pode resultar em graves desequilíbrios ecológicos e comprometer a sobrevivência da espécie.

Palavras chave: Pesticidas. Ácido 2,4-diclorofenoxiacético. Glifosato. Peixe-zebra.

ABSTRACT

Doctoral thesis
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

THE IMPACT OF PESTICIDES ON FISH: INITIAL PARAMETERS AND PERSISTENT AND TRANSGENERATIONAL EFFECTS.

Author: Aline Pompermaier
Advisor: Prof. Dr. Leonardo José Gil Barcellos
Passo Fundo, July 31, 2022

The presence of residues of emerging pollutants, such as pesticides, is a reality all over the world. Coming from different sources, such as direct application, leaching from crops or natural degradation processes, their effects on non-target species are still poorly understood. Zebrafish is an excellent model for ecotoxicological studies, as it has high prolificacy, translucent embryos, external fertilization and rapid development. The effects of fish contamination by environmental concentrations were evaluated by exposing the embryos during the period of organogenesis to the most commonly used herbicides, glyphosate (GBH) and 2,4-D (DBH). We used ambient concentrations of 4.8 ug/L for glyphosate and 3.4 ug/L for 2,4-D. In our first article, we evaluated the initial developmental parameters (mortality, hatching, spontaneous movement and heart rate), behavioral parameters (open field test and aversive stimulus) and also the possible mechanisms of action (SOD, CAT and AChE). In our second article, we evaluated the persistent effects of exposure on adult animals through the novel tank and aversity tests and then we performed the reproduction of these animals to verify the transgenerational effects in the F1 generation, with the same analyzes of the F0 (initial development, behavioral parameters and biochemical biomarkers). In the F0 generation, exposure to glyphosate decreased survival, caused hypermobility and anxiolytic behavior, negatively affected larval anti-predatory behavior, and increased acetylcholinesterase activity, whereas exposure to 2,4-D caused only slight hypermobility in larvae and increased acetylcholinesterase activity. In the adult phase, fish exposed to GBH showed hypermobility and their antipredatory reaction was impaired, characterizing a persistent effect. F1 larvae from fish exposed to GBH had behavioral and survival changes, as well as effects on AChE activity and antioxidant enzymes, characterizing a transgenerational effect. As for DBH, the fish did not show persistence of effects in the adult stage, however, they were not able to reproduce, which demonstrates that the exposure affected the perpetuation of the species. These changes observed at all stages of the animals can compromise the perpetuation of the species, the search for partners/food and facilitate the action of predators, which can result in serious ecological imbalances and compromise the survival of the species.

Keywords: Pesticides. 2,4-Dichlorophenoxyacetic acid. Glyphosate. Zebrafish.

1. INTRODUÇÃO

Os recursos hídricos, sejam eles naturais ou artificiais, estão sujeitos a contaminação por poluentes emergentes. A pressão mundial pelo aumento da produção de alimentos, trouxe à tona desafios que impulsionaram o mercado a adotar medidas para garantir cultivos prósperos. Com isso, a demanda por agrotóxicos cresceu e ainda cresce de forma exponencial (1). São muitas as categorias e classes desses compostos, onde podemos destacar os herbicidas, inseticidas e fungicidas. No Brasil, os agrotóxicos mais comercializados são os herbicidas, e ocupando as primeiras posições da lista estão os compostos a base de glifosato e 2,4-D (1).

Os agrotóxicos são utilizados para fazer o controle de ervas daninhas, insetos e fungos que competem com as monoculturas, portanto, são aplicados diretamente no solo ou nas plantas. Com isso, ficam dispostos no ambiente por longos períodos, sujeitos aos processos naturais de degradação e lixiviação pelas chuvas (2,3). Independente da forma de aplicação, o seu destino acaba sendo os corpos d'água (4,5). Além disso, o glifosato, por exemplo, é utilizado para controlar macrófitas aquáticas, sendo assim, acaba por ser aplicado diretamente em rios e lagos.

Espécies aquáticas convivem diariamente com a presença de poluentes em seu ambiente natural (6–8). Os efeitos sobre esses organismos não-alvo vêm sendo estudados nos últimos anos (9,10), mas em sua grande maioria ainda são pouco elucidados. Alterações fisiológicas (11), comportamentais (12) e bioquímicas (13) têm sido observadas em peixes expostos a essas substâncias, contudo estudos a longo prazo que avaliem as consequências de exposições crônicas ainda são escassos.

Considerado como um modelo animal ascendente, o Peixe-zebra vem sendo muito utilizado para estudos ecotoxicológicos. Por ser um pequeno teleósteo, com cerca de 3-4 cm, possuir embriões translúcidos, fecundação externa, alta prolificidade, desova todos os dias, rápido desenvolvimento (14) e, por ter um comportamento robusto (15,16), foi o modelo animal escolhido para a realização desse trabalho.

Diante do alto consumo dos herbicidas glifosato e 2,4-D e considerando que os organismos aquáticos estão expostos a essas substâncias por longos períodos, decidimos expor os peixes durante todo o período de organogênese (3 – 120 hpf) e avaliar na geração F0 os parâmetros iniciais de desenvolvimento (mortalidade, eclosão, movimentação espontânea e frequência cardíaca), o comportamento (teste do campo aberto e estímulo aversivo) e os parâmetros bioquímicos (SOD, CAT e AChE). Na fase adulta, avaliamos o efeito persistente da exposição em testes comportamentais (teste do tanque novo e aversividade) e na geração F1 os possíveis efeitos transgeracionais com o mesmo protocolo utilizado na F0.

A presente tese está dividida em resumo, introdução ao estudo proposto e os resultados obtidos nos experimentos laboratoriais e as discussões acerca destes, nos capítulos 1: "*Impaired initial development and behavior in zebrafish exposed to environmentally relevant concentrations of widely used pesticides*" e 2: "*Persistent and transgenerational effect of pesticide residues in zebrafish*", por fim são apresentadas as conclusões do estudo e as considerações finais.

2. REVISÃO DE LITERATURA

2.1 Contaminação ambiental

Os recursos hídricos são os receptores dos resíduos químicos e biológicos provenientes das cidades e do campo. A carga desses compostos despejados diariamente sobre esses mananciais é maior que a sua capacidade de depuração. Os contaminantes emergentes são produtos químicos sintéticos e são a principal causa da contaminação dos corpos hídricos (17). Como componentes principais deste grupo, podemos destacar os fármacos, os compostos usados em produtos de higiene pessoal, os hormônios, as drogas ilícitas, os adoçantes artificiais, os agrotóxicos, as nanopartículas, as dioxinas, os microplásticos, entre outros (18).

Esses compostos químicos sintéticos possuem cadeias químicas complexas e por isso se tornam componentes difíceis de serem degradados pelos sistemas de tratamento de efluentes públicos (19,20). Além disso, algumas propriedades físico-químicas desses compostos são persistentes e voláteis, o que acaba por garantir a sua permanência nos ambientes aquáticos por muitos anos (18).

A presença dos agrotóxicos em rios e lagos é conhecida e estudada há mais de 25 anos (21) e hoje já se sabe que é devido à aplicação direta para controle de macrófitas, descarte incorreto de embalagens, lavagem de tanques de aplicação em rios/lagos, lixiviação das lavouras e até mesmo por processos naturais de degradação. No entanto, os seus efeitos sobre os organismos não-alvo que vivem nesses locais ainda são pouco elucidados.

Os agrotóxicos são contaminantes ambientais, contudo, para o setor agrícola eles possuem uma função fundamental na proteção das culturas contra as chamadas pragas agrícolas, seja através da inibição do controle das ervas daninhas, no caso dos herbicidas ou controlar a presença de fungos, função realizada pelos fungicidas ou pelo controle dos insetos, realizado pelos inseticidas. São muitas as classes desses compostos, assim como o seu potencial tóxico. Atualmente, as substâncias mais comercializadas no Brasil são os herbicidas e como princípios químicos em destaque temos os produtos à base de glifosato e 2,4-D (1).

Esse mesmo cenário de comercialização observado no Brasil também é a realidade em outros países, e com isso, é inevitável a presença desses dois compostos em rios e lagos ao redor do mundo (5,22–24). As concentrações ambientais detectadas desses compostos variam muito e podem ser justificadas pelo tipo de contaminação e, também, pela meia-vida desses compostos que acaba variando bastante, mas podemos dar destaque e até mesmo atribuir como principais faixas de detecção os valores em µg/L (25) e mg/L(26).

O Roundup® (Monsanto Co., St. Louis, MO, USA) tem como principal ingrediente ativo o glifosato, e é um herbicida pós-emergente, de amplo espectro, sistêmico, não seletivo do grupo químico glicina substituída. É absorvido pelas folhas das plantas e atua sobre a atividade enzimática inibindo a fotossíntese, a síntese dos ácidos nucleicos e estimulando a produção de etileno (27). O 2,4-D Amina 840 SL® (Albaug Agro Brasil Ltda, SP, BR) é um herbicida pós-emergência, seletivo e sistêmico com base no grupo químico ácido diclorofenoxiacético. O 2,4-D inibe a fosforilação oxidativa das plantas (28).

2.2 O impacto dos agrotóxicos nos peixes

Conforme observamos no item anterior, a contaminação ambiental por resíduos de agrotóxicos é uma realidade e os efeitos dessa contaminação vêm sendo estudados nos últimos anos. Os peixes são animais sensíveis à poluição e considerados como bioindicadores da qualidade das águas. Através da sua presença, dominância, funções fisiológicas ou de desempenho podem nos mostrar o estado do córrego onde vivem (29). Além disso, alterações na água podem ter impacto direto e inevitável nas células, tecidos e órgãos dos peixes (30).

O impacto dos agrotóxicos nos peixes tem sido investigado tanto nos embriões e larvas quanto nos peixes juvenis e adultos. No quadro 1 são demonstrados esses efeitos por substância e espécie.

Quadro 1: Efeito dos agrotóxicos nos peixes

Substância	Espécie	Fase da vida	Efeito observado	Referência
EFEITOS FISIOLÓGICOS				
Glifosato	*Oryzias javanicus*	**Embrionária e larval**	Anomalia no desenvolvimento, eclosão e sobrevivência diminuída	(31)
	Danio rerio		Perda de ventrículos cerebrais delineados e reduções nas regiões cefálica e ocular	(32)
			Atrasos no desenvolvimento, morte embrionária e danos aos axônios CaP	(33)
			Inibição da enzima anidrase carbônica que desencadeou apoptose celular e causou malformações incluindo edema pericárdico, edema do saco vitelino, curvatura da coluna vertebral e malformações	(34)
	Rhamdia quelen		Alterações no sistema antioxidante e efeitos neurotóxicos	(35)
	Danio rerio		Diminuição da distância ocular	(36)
	Odontesthes humensis		Redução do tamanho dos olhos e mortalidade	(37)
	Austrolebias nigrofasciatus		Redução da fertilidade e da tolerância térmica e alteração no desenvolvimento embrionário	(38)
	Danio rerio		Efeitos na eclosão, frequência cardíaca, indução de malformações e aumento da mortalidade	(39)
			Problemas na embriogênese	(40)
	Oryzias latipes		Efeitos no desenvolvimento e reprodução	(41)
	Danio rerio		Cardiotoxicidade e alteração na expressão gênica	(42)
			Aumento da atividade da AChE	(11)
			Eclosão prematura	(43)
	Rhamdia quelen		Redução das taxas de eclosão e fertilidade	(44)
	Rhamdia quelen, Prochilodus lineatus, Danio rerio, Jenynsia multidentata	**Adulta**	Inibição da AChE	(45–47)
	Prochilodus lineatus		Genotoxicidade para brânquias e eritrócitos	(48)
	Danio rerio		Redução da fertilidade masculina	(49)

	Rhamdia quelen, Danio rerio		Redução da fertilidade feminina	(50,51)
Glifosato	*Poecilia vivípara*		Prejuízo na reprodução	(52)
	Colossoma macropomum		Histopatologia, aumento dos índices hematológicos, estresse oxidativo, danos ao DNA e inibição da AChE	(53)
	Austrolebias nigrofasciatus		Casais produziram menos embriões, e os embriões que produziram tiveram tamanho maior que o normal	(38)
	Danio rerio		Modula os complexos da cadeia respiratória mitocondrial e induz a hiperpolarização mitocondrial	(54)
	Oryzias latipes latipes		Efeitos na epigenética	(41)
	Jenynsia multidentata		Dano histológico ao fígado, brânquias e cérebro	(55)
	Poecilia reticulata		Danos histopatológicos	(56)
	Carassius auratus, Prochilodus lineatus, Anguilla anguilla, Pseudoplatystoma corruscans, Oreochromis niloticus, Labeo rohita		Estresse oxidativo	(46,57–61)
	Cyprinus carpio		Inibição da AChE e estresse oxidativo	(62)
	Rhamdia quelen, Cyprinus carpio, Oreochromis niloticus		Imunossupressão	(60,63,64)
	Poecilia reticulata		Danos no DNA e efeitos mutagênicos	(65)
	Oncorhynchus mykiss		Aumento da AChE	(66)
			Toxicidade transgeracional	(67)
			Mortalidade	(68)
	Oreochromis niloticus		Disfunção metabólica hepática	(69)
EFEITOS COMPORTAMENTAIS				
Glifosato	*Danio rerio*	**Larval**	Alterações na locomoção e no comportamento aversivo	(36)
			Elevação significativa das atividades locomotoras	(33)
	Oncorhynchus mykiss		Efeito intergeracional no comportamento	(67)
	Danio rerio		Hipermobilidade, ansiólise e prejuízo na reação anti-predatória	(11)

	Cyprinus carpio	**Adulta**	Perda de equilíbrio, aumento na frequência de movimentos operculares, natação rápida e saltos, exaustão e letargia, natação vertical e sangramento na base dos globos oculares	(70)
	Danio rerio		Alterações comportamentais e perda de memória	(36)
			Comportamento ansiolítico	(12)
			Prejuízo na reação anti-predatória	(71)
			Prejuízos comportamentais	(54)
EFEITOS FISIOLÓGICOS				
2,4-D	*Danio rerio*	**Embrionária e larval**	Redução da sobrevivência e prejuízo no desenvolvimento do sistema visual e alteração nos circuitos neurais	(72)
			Alteração na expressão gênica e estresse oxidativo	(73)
			Cardiotoxicidade, alteração na expressão gênica e estresse oxidativo	(74)
			Aumento da AChE	(11)
			Diminuição da sobrevivência, malformações (edema pericárdico e do saco vitelino), estresse metabólico e oxidativo e dano hepático	(75)
	Pimephales promelas		Sobrevivência diminuída	(76)
	Rhamdia quelen		Genotoxicidade	(44)
	Cnesterodon decemmaculatus	**Adulta**	Genotoxicidade	(77,78)
	Oreochromis niloticus e *Cyprinus carpio*		Estresse oxidativo	(79)
	Rhamdia quelen		Afeta a atividade da AChE, bem como alguns parâmetros metabólicos e histológicos	(80)
	Poecilia reticulata		Neurotoxicidade	(81)
	Pimephales promelas		Sobrevivência diminuída, diminuição significativa na presença de tubérculos masculinos, as gônadas das fêmeas exibiram estágio de maturação do oócito significativamente deprimido, aumento da gravidade da atresia do oócito e uma presença significativa de um tipo de tecido não identificado	(82)
	Prochilodus lineatus, Carassius auratus		Estresse oxidativo	(83,84)

	Danio rerio		Aumento da AChE, alterações bioquímicas e histopatológicas	(85)
	Catostomus commersonii, Pimephales promelas, Esox lucius, Micropterus salmoides, Pomoxis annularis, Perca flavescens, Sander vitreus		Redução da sobrevivência	(86)
	Danio rerio		Prejuízo na função mitocondrial e no estado oxidativo	(87)
	Astyanax lacustris		Danos ao DNA, cromossomos, micronúcleos e alterações nucleares	(88)
EFEITOS COMPORTAMENTAIS				
2,4-D	*Danio rerio*	**Larval**	Leve hipermotilidade	(11)
			Redução de comportamentos essenciais como a caça de presas	(72)
	Poecilia reticulata	**Adulta**	Diminuição da atividade geral, agrupamento, falta de ar, rotações e saltos bruscos, perda de equilíbrio e cor	(81)
	Perca flavescens		Prejuízo na aprendizagem associativa	(89)
	Hyphessobrycon eques		Mudanças no comportamento (natação/velocidade máxima)	(90)
	Cnesterodon decemmaculatus		Maior tempo no fundo do aquário, lentidão no movimento, reação lenta e natação anormal	(77)
	Danio rerio		Prejuízo no comportamento	(87)
			Prejuízo no comportamento anti-predatório	(71)

2.3 Comportamento animal

O comportamento animal pode ser entendido como uma ponte entre os aspectos moleculares e fisiológicos da biologia e da ecologia. É a ligação entre os organismos e o ambiente (91), o comportamento é a reação do animal frente a algum estímulo interno ou externo. Em função disso, alterações comportamentais têm influência direta na boa condição física e na perpetuação de uma espécie (92,93). Com isso, um repertório comportamental intacto tem consequências para o condicionamento físico, porque impacta na sobrevivência e reprodução dos peixes, tanto no nível individual quanto na população (94).

O comportamento dos animais normalmente é uma das principais vias de detecção da degradação ambiental, pois mudanças em comportamentos chave, como comportamentos sexuais ocorrem de forma mais rápida e em níveis mais pontuais do que alterações nos padrões reprodutivo e no tamanho de uma população (91).

O peixe-zebra é um animal muito social (95). Portanto, ao estar em contato com substâncias químicas que interfiram nesse padrão de comportamento, os efeitos para a espécie podem ser muito significativos onde a contaminação pode impactar na busca por alimento, por parceiros sexuais, na interação sexual, na fuga de predadores e na locomoção, o que pode comprometer a vida do animal e a sua perpetuação.

2.4 Efeitos persistentes e transgeracionais

A contaminação da água por substâncias químicas pode impactar os organismos não-alvo de forma direta e pontual. Contudo, algumas formas de contaminação podem não causar efeitos instantâneos, mas sim a longo prazo e impactando não apenas os animais que entraram em contato com a substância, mas as gerações subsequentes também (96,97).

Presume-se que as mudanças epigenéticas sejam a causa dos efeitos transgeracionais (98). Portanto, essas alterações podem ser explicadas pela memória do estado epigenético que não é apagada no processo de reprogramação que ocorre durante a gametogênese e a embriogênese. Com isso, é possível a permanência de alguma memória do estado epigenético que persiste em outras gerações (99).

Essa herança transgeracional da contaminação por produtos químicos foi relatada inicialmente em ratos (100), mas hoje já se tem pesquisas em outros modelos animais, inclusive nos peixes. A exposição ao bisfenol A (BPA) em peixes-zebra causou retração ovariana e reduziu a fertilidade das fêmeas e a taxa de sobrevivência da prole, e efeitos semelhantes nos machos, e os descendentes dos peixes tratados (gerações F1 a F3) demonstraram anormalidades (98). A exposição ao BPA e ao 17α-etinilestradiol (EE2) induz fenótipos transgeracionais de comprometimento reprodutivo e comprometimento da sobrevivência embrionária nas gerações F2 e F3 de peixes expostos durante a fase inicial de desenvolvimento (101).

Ainda, podemos observar estudos que demonstram os efeitos transgeracionais da exposição aos hormônios androgênios em peixes-zebra (102), a toxicidade do benzo[a]pireno na F3 em *Oryzias latipes* (103), os efeitos do inseticida azociclotina em peixes-zebra induzindo a disrupção endócrina inter e transgeracional (104), o inseticida permetrina induzindo mudanças comportamentais transgeracionais ligadas a alterações transcriptômicas e

epigenéticas em peixe-zebra (105). Também encontramos relatos da toxicidade por interferência da melanina e a toxicidade transgeracional do salicilato de etilhexil de filtro UV orgânico em peixe-zebra (106), a metanfetamina induzindo distúrbios de comportamento e toxicidade transgeracional em *Oryzias latipes* (107), os efeitos do salicilato de etilhexil na F1 em peixes-zebra e a atrazina causando efeitos reprodutivos nos netos de avós expostos em *Oryzias latipes* (108).

2.5 Biomarcadores bioquímicos

Os efeitos dos contaminantes ambientais podem ser sinalizados por alterações em enzimas como a acetilcolinesterase (AChE) e em enzimas do sistema antioxidante como as da primeira linha de defesa: superóxido dismutase (SOD) e catalase (CAT). Os efeitos comportamentais ocorridos em animais expostos a agentes químicos podem ser explicados por alterações nos parâmetros bioquímicos (109).

A AChE atua na regulação da neurotransmissão colinérgica do sistema nervoso, mantendo os níveis de acetilcolina estáveis por meio de sua hidrólise nas fendas sinápticas (110). Alterações em sua atividade, como reduções, demonstram excitabilidade do sistema colinérgico devido ao aumento da permanência da ACh na fenda sináptica. Por outro lado, o aumento da atividade da AChE reduz a permanência da ACh na fenda sináptica, reduzindo a eficiência das sinapses e resultando em alterações do comportamento, atenção, cognição e respostas cerebrais naturais. Portanto, a partir dessa importante atividade da AChE na fisiologia dos peixes, ela é um excelente biomarcador para medir a exposição de organismos aquáticos a agrotóxicos (110).

O sistema de defesa antioxidante tem como função inibir ou reduzir os danos causados pela ação deletéria dos radicais livres, ou das espécies reativas de oxigênio (EROS)(111). Durante as funções metabólicas, essas espécies reativas funcionam como intermediários para a transferência de elétrons em diferentes respostas bioquímicas (112). O estresse oxidativo resulta da existência de um desequilíbrio entre compostos oxidantes e antioxidantes, em favor da produção exagerada de espécies reativas ou em detrimento da velocidade de sua remoção (111). Esse sistema é subdividido em enzimático e não enzimático. Aqui avaliamos enzimas do sistema enzimático, com foco nas de primeira linha de defesa do organismo, SOD e CAT.

Essas enzimas agem através de mecanismos de prevenção, impedindo e controlando a formação de radicais livres e espécies não radicais que estão envolvidos com a iniciação das reações que vão culminar com propagação e amplificação do processo e podem levar a

ocorrência de danos oxidativos (111). A primeira enzima a participar do processo é a enzima SOD que dismuta o radical superóxido (O2•-) em peróxido de hidrogênio (H2O2) podendo ser mitocondrial (Cu) e citoplasmática (Zn). Enquanto a CAT é uma enzima que catalisa a redução direta do H2O2 em água (H2O) e oxigênio molecular (O2) (113), ou seja ela age logo após a SOD. A avaliação dessas enzimas, para verificar os efeitos toxicológicos da exposição a agrotóxicos, pode mostrar os efeitos deletérios causados por esse contaminante no metabolismo celular dos peixes.

2.6 Modelo animal

O peixe-zebra, *Danio rerio* (Hamilton, 1822), *zebrafish* ou até paulistinha, é um pequeno teleósteo de 3-4cm, da família Cyprinidae. É nativo do sudoeste da Ásia, onde é encontrado em rios calmos e rasos e plantações alagadas de arroz (114). Por suas inúmeras vantagens vem ganhando muito espaço nos laboratórios ao redor do mundo. Contudo, sua história se iniciou na década de 60, com o biólogo norte-americano George Streisinger, da Universidade do Oregon. Ele utilizava o peixe-zebra para selecionar linhagens que permitissem entender como defeitos em diferentes genes afetavam o desenvolvimento. Contudo, a ascensão ao peixe-zebra só veio em 1981 quando o pesquisador conseguiu publicar um artigo na revista *Nature* (114).

A partir disso, o peixe-zebra tem sido usado em inúmeros estudos envolvendo as mais diversas áreas do conhecimento, como, fisiologia, genética, toxicologia, embriologia, metabolismo, sistema cardiovascular e oncologia (115). Em 2013, ocorreu um avanço para a pesquisa com o peixe-zebra, o seu sequenciamento genético, a partir disso descobriu-se a sua similaridade genética com vertebrados que passa de 70% (115). Essa descoberta, aliada ao fato da estimulação do eixo neuroendócrino desses peixes culminar com a liberação do cortisol como principal glicocorticoide (116,117), torna o zebra um modelo translacional muito apropriado para o estudo do estresse em humanos (118).

Na área da toxicologia, este modelo animal tem sido muito utilizado nos últimos anos, principalmente na avaliação dos efeitos dos poluentes emergentes em diferentes abordagens, tanto fisiológicas (11,119), comportamentais (12,120) quanto bioquímicas (13), além de diferentes classes como fármacos (121,122) e agrotóxicos (123). Essas investigações fornecem respostas muito robustas quanto ao impacto da contaminação das águas por resíduos de substâncias. Onde essa contaminação pode impactar diretamente a vida do animal, tanto em níveis de sobrevivência, podendo afetar a perpetuação da espécie (124), quanto efeitos comportamentais que podem levar a um desequilíbrio ecológico em comportamentos chave

como as reações anti-predatórias (71), como impactos na busca por alimento e parceiros para reprodução (122).

Os embriões e as larvas de peixe-zebra são um universo a parte da espécie, com inúmeras vantagens que incluem desova todos os dias, alta prolificidade, fecundação externa, embriões translúcidos e com rápido desenvolvimento permite o acompanhamento de todos os processos iniciais de desenvolvimento dos animais (14). Com apenas três dias os embriões já eclodiram e no sétimo dia demonstram um amplo leque de comportamentos típicos da espécie (14). Por essa série de vantagens, esse modelo animal foi escolhido para a realização desse estudo.

3. CAPÍTULO 1

Impaired initial development and behavior in zebrafish exposed to environmentally relevant concentrations of widely used herbicides

O artigo intitulado *Impaired initial development and behavior in zebrafish exposed to environmentally relevant concentrations of widely used herbicides* foi aceito para publicação na revista *Comparative Biochemistry and Phisiology, Part C* no dia 09 de Março de 2022, ISSN 1532-0456 (Qualis A2 em Medicina Veterinária e Ciências Ambientais e fator de impacto de 4,52). O artigo foi anexado no formato publicado pela revista.

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Impaired initial development and behavior in zebrafish exposed to environmentally relevant concentrations of widely used pesticides

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ABSTRACT

Pesticides reach water bodies through different routes, either owing to incorrect packaging disposal, direct application to control macrophytes, leaching from fields, or natural degradation processes. In the aquatic environment, adverse effects in non-target species that come in contact with these substances are poorly understood. Currently, the most used pesticides are glyphosate (GBH) and 2,4-dichlorophenoxyacetic acid-based herbicides (DBH), as its presence in water bodies is already known, we used environmental concentrations and our exposure time comprised the entire period of organogenesis (3-120 h post-fertilization). We evaluated the response of embryos in their early development with the parameters of mortality, hatching, spontaneous movement, and heart rate; and it's through behavior the open field test and aversive stimulus, as well as biochemical analyzes of acetylcholinesterase activity (AChE), catalase (CTL) and superoxide dismutase (SOD) as a possible mechanism of action. Exposure to GBH decreased survival, caused hypermobility and anxiolytic behavior, negatively affected the anti-predatory behavior of the larvae, and increases acetylcholinesterase activity, whereas exposure to DBH caused only slight hypermobility in the larvae and increases acetylcholinesterase activity. These changes may compromise the perpetuation of the species, the search for partners/food, and facilitate the action of predators, which can result in serious ecological consequences.

1. Introduction

There is a wide range of substances available on the world market to increase the productivity of agricultural crops, mainly monocultures. Whether these are herbicides, insecticides, or fungicides, the objective is the same, i.e., to control agricultural weeds, insects, and fungi. In Brazil, in recent years, there has been a considerable increase in the registration and approval of pesticides, ranging from 277 in 2016 to 493 records in 2020 (MAPA, 2021). However, only 0.3% of the pesticides applied reaches the target species, while 99.7% are disposed of in the environment (Felix et al., 2019; Pimentel, 1995). Thus, the contamination of water bodies by pesticide residues is a Brazilian reality and also worldwide (Marchesan et al., 2010; Tsaboula et al., 2016; Fernandes et al., 2019; Okada et al., 2020).

Currently the most commonly used class of chemical compounds are glyphosate (GBH)- and 2,4-dichlorophenoxyacetic acid (2,4-D)-based herbicides (DBH) (IBAMA, 2018). Roundup® (Monsanto Co., St. Louis, MO, USA) is a post-emergence, nonselective systemic herbicide based on glyphosate, and belonging to the substituted glycine chemical group (CAS number 1071-83-6). The 2,4-D Amina 840 SL® (Albaug Agro Brasil Ltda, SP, BR) is a post-emergence, selective, and systemic herbicide based on the dichlorophenoxyacetic acid chemical group (CAS number 94-75-7).

Behavioral and physiological effects of the impacts of pesticides on fish have been reported in recent years. In particular, with exposure to glyphosate causing neurotoxicity (Roy et al., 2016), changes in

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morphology and behavior (Bridi et al., 2017), developmental delay, embryonic death, damages CaP axons of embryos, increased locomotor activity of larvae (Zhang et al., 2017) and cardiotoxicity (Gaur and Bhargava, 2019).

Regarding 2,4-D, cardiotoxicity and oxidative stress (Li et al., 2017), impairment of essential behaviors visually guided larvae of fish (Dehnert et al., 2019), reduction larval survival, malformations, oxidative stress and hepatotoxicity (Martins et al., 2021), alteration in mitochondrial metabolism, antioxidant status and innate behavior of zebrafish (Thiel et al., 2020).

An intact behavioral repertoire is crucial for individual and population fitness since it affects both the survival and reproduction of fish (Stewart et al., 2013). Some behavioral changes can be explained by changes in biochemical parameters, as for example the increase in acetylcholinesterase (AChE) activity explaining fish hyperactivity (Xie et al., 2015). AChE actuates on the muscular junctions and in the synaptic clefts of cholinergic neurons where catalyzes the neurotransmitter acetylcholine (ACh) to acetate and choline. Reductions in its activity had shown excitability of the cholinergic system due to the increase of the permanence of the ACh on the synaptic cleft. On the other hand, the increased AChE activity reduces the permanence of the ACh on the synaptic cleft reducing either the efficiency of the synapses resulting in alterations of the behavior, attention, cognition and natural cerebral responses. Due to this important activity of AChE in fish physiology, it is an excellent biomarker for measuring the exposure of aquatic organisms to pesticides (Santana et al., 2021).

Zebrafish (*Danio rerio*) is a good and high throughput research model for aquatic ecotoxicology studies (Howe et al., 2013), with a robust behavior (Gerlai, 2014; Kysil et al., 2017) and many advantages, especially in its embryonic and larval stages owing to external fertilization, daily spawning, high prolificacy, transparency of the chorion, and translucency of its embryos, allowing easy visualization of internal processes, such as the formation and function of internal organs within the living animal, has been widely used in the last decades. In addition, the fish has rapid development; on the third day they have hatched, and on the seventh day they show typical behaviors of the species (Dahm and Geisler, 2006).

In our previous study, we found that acutely exposed to residual concentrations of GBH and DBH compromised the anti-predatory reaction of adult fish (Pompermaier et al., 2020). However, the effects of chronic exposure to environmental concentrations of these pesticides in zebrafish embryos remain poorly understood. Based on the current literature and in our former papers, here we describe the effects of GBH and DBH on the initial developmental parameters (mortality, hatching, spontaneous movement, and heart rate), larval behavior (open field test and aversive stimulus), and biochemical implications (acetylcholinesterase activity - AChE, catalase -CTL and superoxide dismutase -SOD) in zebrafish.

2. Materials and methods

2.1. Ethical and legal note

This study complies with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Commission for Animal Use Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #0016/2019 - CEUA). In addition, was registered in SisGen (Sistema Nacional de Patrimônio Genético e do Conhecimento Tradicional Associado) and complied with their guidelines (registration code A14E252).

2.2. Study strategy

To evaluate the effects of the agrichemicals GBH and DBH on the initial development of zebrafish, we exposed the embryos to environmentally relevant concentrations of GBH (4.8 μg/L) and DBH (3.4 μg/L) in the organogenesis phase (3–120 h post-fertilization) to quantify the initial development parameters, the exploratory and anti-predatory behavior of the larvae, and the possible mechanisms of action (Fig. 1).

2.3. Reproduction and maintenance of embryos

For breeding, we selected healthy wild-type zebrafish, aged between 3 and 18 months. Couples were placed in the afternoon in breeding tanks separated from the bottom to prevent egg predation. The next morning, the embryos were collected by siphoning and washed to remove debris and feces. Embryos were sorted and maintained in 24-well cell culture plates (3 mL/well), with 10 embryos per well and incubated in a 28 °C water bath to 7 days post-fertilization (dpf). Embryos were maintained in E3 medium (reverse osmosis water +60 mg/L Ocean Tech Bio Active®, Hong Kong, China) (Buske and Gerlai, 2012) with oxygen concentrations at 6.2 ± 0.4 mg/L, pH 7.0 ± 0.2, total ammonia at <0.01 mg/L, total hardness at 6 mg/L alkalinity at 22 mg/L $CaCO_3$ and natural photo period (14 h light, 10 h dark). We performed 5 reproductions to obtain the embryos needed for analysis. In each reproduction, 5 tanks were used with 7 couples in each tank. For the tests, embryos of up to 3 h post-fertilization (hpf) were used (Kimmel et al., 1995).

2.4. Agrichemicals and concentrations tested

The concentrations of GBH and DBH have previously been identified in natural water bodies; for GBH, we used a concentration of 4.8 μg/L (Okada et al., 2020) and for DBH, the concentration used was 3.4 μg/L (Marchesan et al., 2010). We used these concentrations to address the effects of the lower concentrations detected in the environment. We opted for only one concentration of each pesticide, as our goal is to verify the possible effects of environmentally relevant concentration. The products were purchased commercially, Roundup® and 2,4-D Amina 840 SL were used for GBH and DBH, respectively. All the products used were new and with concentrations guaranteed by the manufacturers and by law (BRASIL, 1989). The solutions were prepared serially way, starting from 1 g/L to 0.01 g/L until reaching the environmental concentration tested. After preparation, they were stored in amber glass bottles, where they remained warm in a water bath to be used when necessary. A new mother solution was made at each reproduction and at 72 hpf the solution was refreshed. The 3rd day was chosen because the embryos had already hatched, and it was necessary to remove the remaining dirt from the egg. The embryos were exposed to GBH and DBH from 3 hpf to 120 hpf (Kalichak et al., 2017). The entire period of fish organogenesis occurs in this phase, a crucial period in their development, so we consider our exposure to be chronic. In all tests

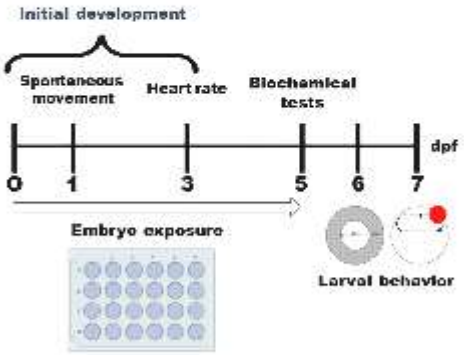

Fig. 1. Schematic representation of study design.

performed, embryos and larvae were randomly chosen. Furthermore, the animals were not repeated in the evaluations.

2.5. Developmental and survival parameters

2.5.1. Survival and hatching analysis

Larvae were analyzed every morning for 7 d to assess mortality and until the 3rd day for hatching. Embryos and larvae that lacked transparency, were clotted, or without cell formation, cardiac movement, or blood circulation were considered dead and removed. Embryos that ruptured the chorion or outer membrane of the egg were considered hatched. Exposure of all, or most, of the tail out of the egg was also classified as hatched. Analysis of mortality and hatching was performed through monitoring 15 wells with 10 embryos per well, totaling 150 embryos per group.

2.5.2. Spontaneous movement

In 24 hpf the embryos present spontaneous movements of the tail still inside the chorion. Spontaneous movements are induced by the development of the motoneurons without any control by the central nervous system (Fraysse et al., 2006; Jin et al., 2009; Kimmel et al., 1995). These movements were recorded for 60 s in 30 embryos per group, using a stereomicroscope (Nova Instruments, Piracicaba, Brazil).

2.5.3. Heart rate

At 72 hpf, the count of the heart rate of the embryos was evaluated for 60 s in 20 larvae, per group using Nikon E-100 binocular biological microscope (Kalichak et al., 2017). In this phase, all embryos show cardiac activity and little locomotor activity.

2.6. Behavioral analysis

2.6.1. Open field test (OFT)

Larvae (6 dpf) were individually placed in 6-well cell culture plates with a total capacity of 15 mL, filled with 10 mL of E3 medium, and filmed for 7 min (1 min acclimatization +6 min exploration) (adapted from Kalichak et al., 2019). To evaluate behavioral variables in each period, we filmed 16–26 larvae by a group. We used the camera function of the smartphone iPhone 6s iOS 14.6 and the videos were analyzed using the automated tracking software ANY-maze® (Stoelting Co., Wood Dale, USA). To analyze thigmotaxic behavior, the test aquarium was virtually divided into a central and peripheral/wall zone. The parameters used to evaluate the behavior of the larvae were distance traveled (m), rotations (also called circling, a 360 change in orientation in the horizontal plane), absolute turn angle (°), line crossings (considering the full body), time in the central zone (s), entries into the central zone, distance traveled in the central zone (m), time in the peripheral zone (s), and distance traveled in the peripheral zone.

2.6.2. Aversive stimulus test (AST)

The AST was used to assess the ability of the larvae to identify possible predator menaces and move away from this risk area. To perform AST, we placed the 7-dpf larvae in 6-well cell culture plate with a total capacity of 15 mL, filled with 10 mL of E3 medium, at a density of 5 larvae per well with 15 replicates ($n = 75$ per group). Then, we placed the plates above an LCD monitor and, after 2 min of the adaptation, started exposure to the visual stimulus consisting of a red sphere of 1.35 cm with a trajectory that covered only half the well (Bridi et al., 2017; Pelkowski et al., 2011). The sphere movement was made using PowerPoint software (Microsoft Office Professional Plus 2016) and larvae were exposed for 5 min to this aversive stimulus. At the end of the test, we counted the number of larvae that remained in the stimulus area (Kalichak et al., 2017). The larvae were filmed using the camera function of the smartphone iPhone 6s iOS 14.6.

2.7. Biochemical tests

At the end of the exposure (5 dpf), the larvae were euthanized with ice-cold water (Leary et al., 2013), washed, and collected for biochemical analysis acetylcholinesterase (AChE), catalase (CTL), and superoxide dismutase (SOD). We used 20 larvae per pool with four pools per group. The entire body was homogenized to quantify the enzymes. The 20-larvae samples were frozen in liquid nitrogen and kept in an ultra-freezer at −80° until analysis.

2.7.1. Protein determination

Protein determination was performed according to the method described by Bradford (1976), using bovine serum albumin as the standard and Coomassie bright blue as the colorimetric reagent. The absorbance of each sample was quantified at a wavelength of 595 nm and calculated according to a known standard protein curve.

2.7.2. Acetylcholinesterase (AChE) activity

AChE activity was evaluated as described by Ellman et al. (1961). The tissue of the entire body of the larvae was used owing to their small size and was homogenized in potassium phosphate buffer (100 mM pH 7.0) for 1 min in an ultrasonic mixer on an ice bath. Next, the extract was centrifuged for 10 min at 13000 ×g to 4 °C, and the supernatant was collected for use in the enzymatic test. Before adding the substrate acetylcholine (Ach, 8%), the enzymatic sample was pre-incubated for 5 min at 25 °C with Ellman's reagent (DTNB, 100 mM, pH 7,0). The reaction was initiated with the addition of the substrate of ACh and the absorption was measured at a wavelength of 412 nm for 5 min (at 30 s intervals). The activity was expressed in μmol of AChI/h/mg protein.

2.7.3. Catalase (CTL) and superoxide dismutase (SOD) activity

The same homogenate was used to evaluate CTL and SOD activity. Measurement of CTL activity was performed as described by Johansson and Borg (1988) and Góth (1991). For CTL, the spectrophotometer was zeroed with 10 μL of homogenate (20–30 μg of protein) in a quartz cuvette and 1000 μl of 50 mM potassium phosphate buffer (pH 7.5). After that, 50 μL of hydrogen peroxide (0.3 M) was added. The reaction was quantified every 30 s for 2 min at a wavelength of 240 nm and expressed as U of CTL/mg protein. SOD activity was measured as described by Misra and Fridovich (1972) and Müller et al. (2018). The protein content was determined according to the method established by Bradford (1976). For SOD, was initiated by adding 50 uL a sample (100 μg of protein) on 950 uL of glycine buffer (50 mM, pH 10.5) and the enzymatic reaction was started with the addition of epinephrine (60 mM, pH 2.0). The colorimetric reaction was measured at 480 nm and expressed as U of SOD/mg protein.

2.8. Statistics

Survival and hatchability data were evaluated using the Kaplan–Meier method. For the analysis of the data on heart rate, spontaneous movement, OFT, AST, and biochemical tests, the unpaired *t*-test or Mann–Whitney test (for data not normally distributed according to Bartlett's test) were used. All groups were compared to the control group. The alpha level was set to 0.05. All statistical analyses were performed using GraphPad Prism® version 6.07 (GraphPad Software, San Diego, USA).

3. Results

3.1. GBH

3.1.1. Development parameters

GBH exposure impaired embryo survival rate but did not affect hatching, spontaneous movement, or heart rate (Table 1).

Table 1
Developmental parameters of zebrafish larvae exposed to glyphosate-based herbicides (GBH).

Parameter	Control	GBH	Statistics
Survival rate (%)[a]	91.22	87.26	$\chi^2 = 5.003$, $P = 0.0253$*
Hatching (%)[b]	66.33 ± 34.67	68 ± 35.91	$t = 0.03339$, $P = 0.9750$
Spontaneous movements[b]	2.367 ± 0.2372	1.967 ± 0.2116	$t = 1.258$, $P = 0.2134$
Heart rate[b]	137.8 ± 4.109	143.1 ± 4.109	$t = 1.081$, $P = 0.2864$

[a] Survival rates were compared using Kaplan–Meier survival curves.
[b] Unpaired *t*-test. Data are expressed as mean ± S.E.M.
* denotes statistical differences ($P < 0.05$).

3.1.2. Behavioral tests

3.1.2.1. Open field test (OFT). Exposure to GBH increased the number of rotations of the larvae ($t = 2.705$; $P = 0.0112$; Fig. 2B), line crossings ($t = 2.084$; $P = 0.0457$; Fig. 2D), the time spent in the central zone (U = 56; $P = 0.0058$; Fig. 2E), entries into the central zone ($t = 2.148$; $P = 0.0399$; Fig. 2F), and the distance traveled in the central zone (U = 66.50; $P = 0.0194$; Fig. 2G), but decreased the time spent at the periphery (U = 64; $P = 0.0152$; Fig. 2H). There was no difference in the total distance covered ($t = 0.3730$; $P = 0.7118$; Fig. 2A), absolute turn angle ($t = 1.696$; $P = 0.1002$; Fig. 2C), or the distance traveled in the peripheral zone ($t = 0.6223$; $P = 0.5385$; Fig. 2I).

3.1.2.2. Aversive stimulus test (AST). A higher number of larvae from the group exposed to GBH than from the control remained in the stimulated area (U = 53; $P = 0.0291$; Fig. 3).

3.1.3. Acetylcholinesterase (AChE) and antioxidant enzymes

The larvae exposed to GBH showed increased activity of AChE, while did not change the levels of the enzymes SOD and CTL (Table 2).

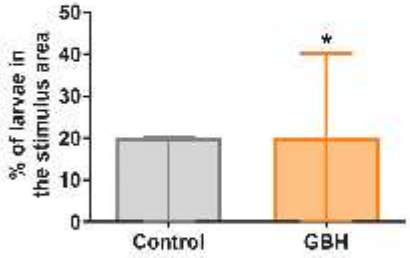

Fig. 3. Aversive stimulus test in fish exposed to glyphosate-based herbicides (GBH). The data were compared using the Mann–Whitney test and are expressed as median ± interquartile range (*$P < 0.05$).

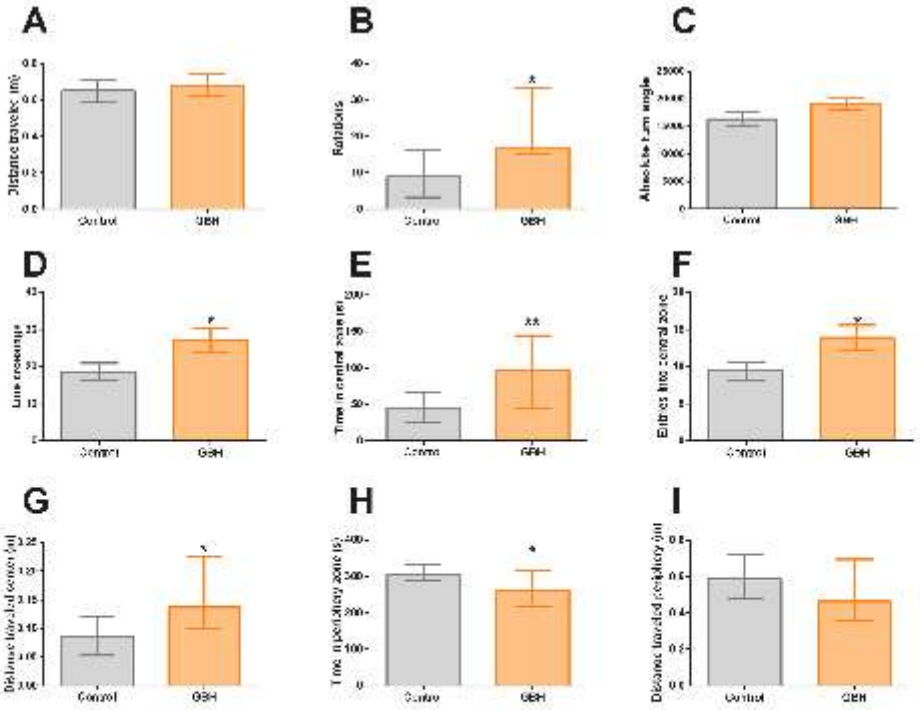

Fig. 2. Open field test results for fish exposed to glyphosate-based herbicides (GBH). (A) Distance traveled (m), (B) rotations, (C) absolute turn angle, (D) line crossings, (E) time in central zone (s), (F) entries into the central zone, (G) distance traveled in the central zone (m), (H) time in the peripheral zone (s), and (I) distance traveled in the peripheral zone (m). The data in panels A, B, C, D, F and I were compared using an unpaired *t*-test and are expressed as mean ± S.E.M. The data in panels E, G and H, were compared using a Mann–Whitney test and are expressed as median ± interquartile range (*$P < 0.05$, **$P < 0.01$).

Table 2
Acetylcholinesterase and antioxidant enzymes in zebrafish larvae exposed to glyphosate-based herbicides (GBH). Acetylcholinesterase activity (AChE) expressed in μmol/h/mg of protein; superoxide dismutase activity (SOD) expressed in U of SOD/mg of protein; catalase activity (CTL) expressed in mmol/min/mg of protein.

Parameter	Control	GBH	Statistics
AChE[a]	0.0078 ± 0.00038	0.0090 ± 0.00304	$t = 2.535$; $P = 0.0189$*
SOD[b]	0.7066 ± 0.2039	0.6547 ± 0.1133	$t = 0.2328$, $P = 0.8183$
CTL[b]	0.0370 ± 0.03064	0.0247 ± 0.01019	U = 47.00, $P = 0.2604$

SOD, superoxide dismutase; CTL, catalase.
[a] Unpaired *t*-test. Data expressed as mean ± S.E.M.
[b] Mann–Whitney test. Data expressed as mean ± S.E.M.
* denotes statistical differences ($P < 0.05$).

3.2. DBH

3.2.1. Initial parameters

Exposure to DBH did not affect spontaneous movement, survival rate, hatching, or heart rate (Table 3).

3.2.2. Behavioral tests

3.2.2.1. Open field test (OFT). Exposure to DBH increased the number of rotations of the larvae ($t = 2.380$; $P = 0.0222$; Fig. 4B) and absolute turn angle ($t = 2.498$; $P = 0.0167$; Fig. 4C), but did not affect distance traveled ($t = 0.5726$; $P = 0.5701$; Fig. 4A), line crossings ($t = 1.241$; $P = 0.2218$; Fig. 4D), time in the central zone (U = 149.5; $P = 0.1323$; Fig. 4E), entries into the central zone ($t = 1.240$; $P = 0.2221$; Fig. 4F), distance traveled in the central zone ($t = 1.520$; $P = 0.1364$; Fig. 4G), time at the periphery (U = 166.5; $P = 0.2888$; Fig. 4H), and distance traveled in the peripheral zone ($t = 0.2003$; $P = 0.8422$; Fig. 4I).

3.2.2.2. Aversive stimulus test (AST). The number of larvae exposed to DBH (24 ± 6.234 larvae) in the area stimulated at the end of the test did not differ from that in the control group (16 ± 5.237 larvae) (U = 66; $P = 0.1327$).

3.2.3. Acetylcholinesterase (AChE) and antioxidant enzymes

Exposure to DBH increased AChE activity in the larvae, while did not alter the levels of the enzymes SOD and CTL (Table 4). Acetylcholinesterase activity (AChE) expressed in μmol/h/mg of protein; superoxide dismutase activity (SOD) expressed in U of SOD/mg of protein; catalase activity (CTL) expressed in mmol/min/mg of protein.

4. Discussion

Exposure to GBH decreases survival, causes hypermobility and anxiolytic-like behavior, negatively affects the anti-predatory behavior of the larvae and increases acetylcholinesterase activity, whereas exposure to DBH causes only slight hypermobility in the larvae, and increases acetylcholinesterase activity.

Table 3
Developmental parameters of zebrafish larvae exposed to 2,4 dichlorophenoxyacetic acid-based herbicides (DBH).

Parameter	Control	DBH	Statistics
Survival rate (%)[a]	91.22	87.26	$\chi^2 = 3.207$, $P = 0.0733$
Hatching (%)[b]	66.33 ± 34.67	71.67 ± 38.32	$t = 0.1032$, $P = 0.9228$
Spontaneous movements[b]	2.367 ± 0.2372	1.767 ± 0.2116	$t = 1.883$, $P = 0.0647$
Heart rate[c]	137.8 ± 4.109	143.1 ± 2.673	U = 145.5, $P = 0.1420$

[a] Survival rates were compared using Kaplan–Meier survival curves.
[b] Unpaired *t*-test. Data are expressed as mean ± S.E.M.
[c] Mann–Whitney test. Data are expressed as mean ± S.E.M.

In fact, exposure to GBH reduced larval survival, which may result in a smaller number of individuals in each generation, affecting the perpetuation of the species. In addition, GBH caused an increase in the number of rotations and line crossings, characterizing hypermobility and high exploration of the tank. This hypermobility was accompanied by a clear anxiolytic-like behavior, with larvae spending more time and swimming more in the central zone. Under normal conditions, when in a new environment, the zebrafish larvae stay for long periods at the bottom or close to the peripheral areas, and after an acclimation period, they begin to explore the environment gradually (Kysil et al., 2017; Stewart et al., 2012). The anxiolytic effect on the larvae exposed to GBH, resulting in them staying longer in the central zone and having greater mobility, facilitates the action of predators and demonstrates a risk for their survival.

Considering the anxiolytic effect of GBH, exposure to this herbicide may also compromise the anti-predatory reaction of the larvae in the AST. In fact, after exposure to GBH, more individuals remained in the stimulus area. An unexposed normal fish, on detecting the presence of a possible predator, leaves the area of greatest risk and remains close to the periphery, a precautionary behavior called thigmotaxis (Ahmad and Richardson, 2013), that has been reported as a strategy to reduce predation risk (Colwill and Creton, 2011; Treit, 1989). However, the larvae exposed to GBH remained in the risk area and remained hypermobile at the center of the OFT arena, demonstrating the impairment in risk assessment and avoidance capacity. We cannot rule out the possibility that these negative effects of the anti-predatory reaction persisted in the embryos and larvae exposed to GBH, as GBH also compromises this reaction in adult fish (Pompermaier et al., 2020).

Regarding DBH, there was no change in the initial development parameters. In the OFT, the larvae exposed to DBH showed an increase in the number of rotations and in the absolute turn angle, indicating hypermobility and a high level of exploration of the tank, similar to that observed in larvae exposed to GBH. Hypermobility may represent a risk to fish survival because it facilitates its detection by the predator and increases the chances of being predated. However, DBH had no effect on anti-predatory reactions assessed by OFT. With this range of effects demonstrated by the embryos and larvae exposed to GBH and DBH, the question of what mechanism underlies the behavioral changes remains to be verified.

Mechanistically, in both GBH- and DBH-exposed fish, there was an increase in the activity of the AChE enzyme. The increase in AChE activity is the key to the hypermobile behavior observed in GBH- and DBH-exposed larvae (Xie et al., 2015). In our study, the increase in AChE activity may also be related to chronic exposure; and also because the organs responsible for detoxification and elimination of toxic substances (liver and kidney) in the larvae are not fully developed and do not occur at the same rate observed in larger and older fish (Calfee et al., 2014; Mohammed, 2013). The observed increase in AChE can be related to loss of attention due to the reduced controlled command of the nerves. This effect can contribute to the uncontrolled motility observed on larvae (Yoshida et al., 2009). Other observation regarding about the toxicity highlighted for the AChE increase that can serve as a biomarker for the apoptosis process (Kaizer et al., 2010). Therefore, this observed increase in AChE in the larvae exposed to GBH and DBH can alter behavior and compromise fish survival (Santana et al., 2021).

Interestingly, the behavior profiles were different in larvae exposed to GBH and DBH (especially in the AST test), but both increased the AChE activity. In fact, in the OFT, both GBH- and DBH-exposed fish are slightly hypermobile; however, only GBH-exposed larvae remain in the stimulus area in the AST. This OFT and AST data profile suggest that the reduced risk perception of GBH-exposed larvae in AST was not due the hypermobility, reinforcing the hypothesis of the impairment in risk assessment and avoidance capacity. In fact, these different profiles might be related to other GBH and DBH formulae components as the

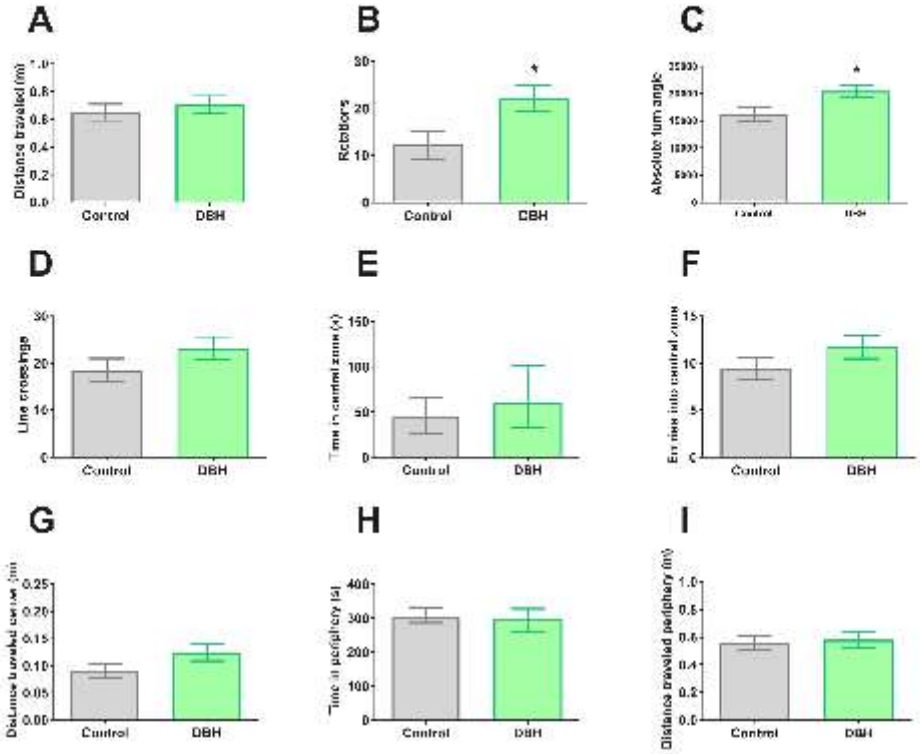

Fig. 4. Open field test results for fish exposed to 2,4-dichlorophenoxyacetic acid-based herbicides (DBH). (A) Distance traveled (m), (B) rotations, (C) absolute turn angle, (D) line crossings, (E) time in the central zone (s), (F) entries into the central zone, (G) distance traveled in the central zone (m), (H) time in the peripheral zone (s), (I) distance traveled in the peripheral zone (m). The data in panels A, B, C, D, F, G, and I were compared using an unpaired *t*-test and are expressed as mean ± S.E.M. The data in panels E and H were compared using the Mann–Whitney test and are expressed as median ± interquartile range (*$P < 0.05$).

Table 4
Acetylcholinesterase and antioxidant enzymes in zebrafish larvae exposed to 2,4-dichlorophenoxyacetic acid-based herbicides (DBH).

Parameter	Control	DBH	Statistics
AChE[a]	0.0078 ± 0.00038	0.0089 ± 0.00023	$t = 2.671$; $P = 0.0139$*
SOD[b]	0.3575 ± 0.3747	0.7929 ± 0.2424	$U = 58.00$, $P = 0.8977$
CTL[b]	0.0370 ± 0.3064	0.0274 ± 0.0146	$U = 51.00$, $P = 0.8094$

AChE, Acetylcholinesterase; SOD, superoxide dismutase; CTL, catalase.
[a] Unpaired *t*-test. Data expressed as mean ± S.E.M.
[b] Mann–Whitney test. Data are expressed as mean ± S.E.M.
* denotes statistical differences ($P < 0.05$).

surfactants used to improve the product distribution over the entire leaf surface. It is even known that the nonionic surfactant polyoxyethylene amine (POEA) from glyphosate is more toxic than the active ingredient and the product itself formulated for aquatic organisms (Tsui and Chu, 2003).

Another candidate mechanism evaluated was the activity of the antioxidant enzymes SOD and CTL; however, no changes were observed in these enzymes in the larvae exposed to GBH and DBH. However, we cannot disregard the eventual oxidative stress induced by GBH and DBH as we did not measure pro-oxidant biomarkers (e.g., thiobarbituric acid reactive substances) and because this eventual oxidative damage may have been reversed given our exposure period of only 5 days. Some studies have reported acute exposure-related oxidative stress induced by both GBH (Modesto and Martinez, 2010; de Moura et al., 2017) and DBH (Oruc et al., 2004).

Another possible mechanism underlying our behavioral results of GBH exposed larvae, is the disruption of hypothalamus-pituitary-interrenal axis (Cericato et al., 2008, 2009; Koakoski et al., 2014). In addition, behavioral and morphological changes at different developmental stages was already verified in GBH-exposed zebrafish (Bridi et al., 2017). GBH is also aversive to zebrafish (da Rosa et al., 2016) and we cannot discard that the fish were unable to concentrate on risk assessment tasks by the GBH-induced pain (Jirasungkoorskul et al., 2002).

The mechanism of action of DBH seems to be related to a possible damage to the central nervous system (CNS). In this line Dehnert et al. (2019) shown an impairment of the visual system function in zebrafish larvae, which can alter the neural circuits responsible for predator escape behaviors. Some behavioral changes were already described in fish exposed to DBH, such as lethargy, erratic swimming (Cattaneo et al., 2008), slowness in motion, slow reactions, and abnormal swimming (de Arcaute et al., 2016). Thiel et al. (2020) report alteration in mitochondrial metabolism, antioxidant status and innate behavior of zebrafish. Based on this, the mechanisms of the DBH impairment on larval behavior are related to effects on the CNS. We did not test these

hypotheses and recognize this as a limitation of our work, however, this does not lessen the relevance of the hypotheses tested herein. Therefore, the behavioral effects observed in the larvae exposed to GBH and DBH may reflect the other mechanisms of action described previously.

Finally, when we take our results together, our main novelty is the impairment of the anti-predatory reaction of larvae exposed to GBH and the increased activity of AChE and hypermobility in fish exposed to both GBH and DBH, which indirectly may also impair the anti-predatory reaction of exposed larvae that become more visible to predator. Our results underscore the importance of the 17 sustainable development goals (SDGs) of the United Nations (UN) which aim to protect the planet for future generations. Specifically here we emphasize the need the guarantee of drinking water and sanitation (SDG 6) and life in the water (SDG 14), as our results highlight the consequences of the exposure of zebrafish embryos and larvae in their early development to pesticides widely used and already detected in the environment, which may compromise relevant parameters for the maintenance of the species such as survival and also adversely impact locomotion, exploratory and anti-predatory behavior. These changes can compromise the perpetuation of the species and, because fish are an important component of the food chain, the changes presented here can interfere with the maintenance of the population, search for partners or food and facilitate the action of predators, which can result in serious ecological consequences.

Ethics approval and consent to participate

This study was approved by the Ethics Commission for Animal Use Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #0016/2019 - CEUA).

Consent for publication

Not applicable.

Availability of data and materials

Data will be made available upon reasonable request.

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CRediT authorship contribution statement

A.P.: Conceptualization, Experimental work, Writing - Original draft preparation.

A.C.C.V.: Experimental work and methodology.

M.T.M.: Experimental work and methodology.

S.M.S.: Experimental work and methodology.

M.F.: Experimental work and methodology.

C.A.: Laboratory analyses.

W.A.T.: Laboratory analyses.

L.J.G.B.: Conceptualization, Supervision Data analysis, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4. CAPÍTULO 2

Persistent and transgenerational effect of pesticide residue in zebrafish

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Persistent and transgenerational effects of pesticide residues in zebrafish

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ABSTRACT

Highly toxic chemical compounds are present in rivers and lakes, endangering the survival of non-target species. To evaluate the effects of environmental contamination on non-target species, we used the zebrafish as an animal model. Environmental concentrations of the widely used pesticides, glyphosate (GBH) at 4.8 $\mu g \cdot L^{-1}$ and 2,4-dichlorophenoxyacetic acid (DBH) at 3.4 $\mu g \cdot L^{-1}$, were used. The animals were exposed during the entire period of organogenesis and evaluated in our previous study regarding initial developmental parameters. In the present study, we evaluate these fish when achieve the adult phase, using the novel tank test (NTT) and the aversivity test. In the second step, the animals were allowed to reproduce, and the initial parameters of development, behavioral parameters in the open field test (OFT) and in the aversivity test (AST), and biochemical biomarkers as acetylcholinesterase (AChE), catalase (CAT), and superoxide dismutase (SOD) in the F1 generation were studied. Fish exposed to GBH showed hypermobility, and their anti-predatory reaction was impaired during adulthood, indicating a persistent effect. We also showed that fish had impaired behavioral and survival changes in the F1 generation as well as effects on AChE activity and antioxidant enzymes, characterizing a transgenerational effect. The fish did not show persistent effects in adulthood due to DBH exposure; however, they were unable to reproduce. Our findings demonstrate the serious impact of pesticides on fish, where the effects of contamination can affect future generations and compromise the species' survival.

1. Introduction

Aquatic environments are the final receptors for many chemicals. In these environments, compounds can biotransform and exhibit changes in their concentration and toxicity. However, some non-target species live in these environments and are affected by this contamination. Fish have been the focus of several studies in recent years because they are sensitive to environmental changes and are excellent biomarkers for aquatic environmental monitoring.

Certain substances have many deleterious effects on species, affecting exposed animals and causing alterations that can be observed in all subsequent generations (Kalichak et al., 2019; Kubsad et al., 2019). Understanding the transgenerational effects of exposure to environmental contaminants may explain the changes in species dynamics. Additionally, long-term contamination may affect animal health, survival, and reproduction.

As a result of exposure to emerging pollutants, both drugs and pesticides, some studies have shown effects on the physiology (Freddo et al., 2021), biochemistry (Tamagno et al., 2022a), and behavior (Chaulet et al., 2019) of fish. Therefore, it is important to monitor the impact of substances with high consumption rates and, consequently, a greater probability of contaminating aquatic environments.

Pesticides are an important class of contaminants, as they contribute to the high productivity of monocultures by combating insects, weeds, and fungi that compete with crops. However, its toxic potential has been investigated, and warnings about its effects on non-target organisms have been raised (da Rosa et al., 2016; Chaulet et al., 2019; Pompermaier et al., 2022). Currently, the most consumed pesticides are glyphosate (GBH) and 2,4-dichlorophenoxyacetic acid (DBH) (IBAMA, 2018). Roundup® (Monsanto Co., St. Louis, MO, USA) is a post-emergence, non-selective systemic herbicide based on glyphosate and belongs to the substituted glycine chemical group (CAS number 1071-

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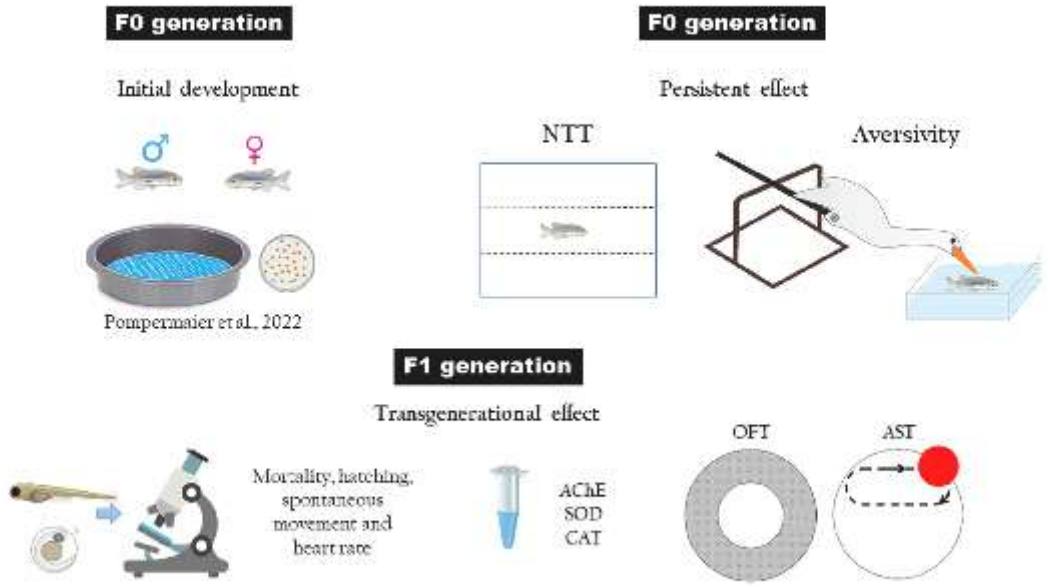

Fig. 1. Schematic representation of study design.

83-6). 2,4-D Amina 840 SL® (Albaug Agro Brasil Ltda, SP, BR) is a post-emergence, selective, and systemic herbicide based on the dichlorophenoxyacetic acid chemical group (CAS number 94-75-7).

Behavior is a response to physiological connections and the environment (Clift et al., 2014; Orger and Polavieja, 2017). An undamaged behavioral repertoire is essential for ensuring the survival and physical fitness of animals (Stewart et al., 2013). Some chemical compounds have altered the behavior of animals (Kalichak et al., 2017). However, changes in biochemical parameters may be the key to these changes in behavior (Xie et al., 2015). The study of certain enzymes, such as acetylcholinesterase (AChE), might explain the effect of pesticides on fish (Santana et al., 2021).

Another important candidate mechanism underlying the behavioral alterations is linked to enzymes that act to reduce oxidative compounds such as superoxide dismutase (SOD) and catalase (CAT). These enzymes compose the first line of antioxidant defenses and are very important in the beginning of the free radical scavenger process. Due to this is important to understand the effect of xenobiotics on these non-target enzymes (Tamagno et al., 2022b).

The zebrafish is an animal model that has gained prominence in scientific research in recent years. It is very versatile and can be used in several studies such as toxicology (da Rosa et al., 2016), pharmacology (Barcellos et al., 2020), immunology (Kirsten et al., 2018), and nutrition (Dametto et al., 2018). Here, we chose this animal model because of its high prolificacy, ease of handling, translucent embryos, and rapid development, which facilitated the execution of the study.

Recent studies have demonstrated the effects of acute (Bridi et al., 2017; Thiel et al., 2020) and chronic exposure (Du-Carrée et al., 2021; Zheng et al., 2021) to pesticides by fish; however, assessments involving more than one generation are still scarce. Therefore, this study aimed to evaluate the persistent and transgenerational effects in exposed animals as embryos (Pompermaier et al., 2022), verifying the behavioral effects in their adult stage and the effects on early development, behavior, and biochemical biomarkers of their offspring (F1 generation).

2. Material and methods

2.1. Ethical and legal note

This study followed the guidelines of the National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Commission for Animal Use Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #0016/2019 - CEUA). In addition, it was registered with SisGen (Sistema Nacional de Patrimônio Genético e do Conhecimento Tradicional Associado) and complied with the guidelines (registration code A14E252).

2.2. Study strategy

Zebrafish embryos and larvae were exposed to environmentally relevant concentrations of GBH and DBH to assess the persistent and transgenerational effects of exposure (Pompermaier et al., 2022). In this study, we subjected the exposed adult embryos (F0 generation) to behavioral tests (novel tank test (NTT) and aversivity). We also reproduced these adults and evaluated the F1 generation for early development, behavior, and biochemical biomarkers (AChE, SOD, and CAT) (Fig. 1).

2.3. Exposure time and pesticides concentrations – F0

Embryos and larvae were exposed to GBH and DBH during the entire period of organogenesis, which comprised 3–120 h post-fertilization (hpf). The concentrations used were 4.8 $\mu g \cdot L^{-1}$ for GBH and 3.4 µg/L for DBH. Embryos were kept in 24-well cell culture plates filled with E3 medium at 28 °C until day 7 and then placed in aquariums with clean water for growth. Further details regarding the exposure and maintenance of embryos and larvae can be found in Pompermaier et al. (2022).

2.4. Reproduction and maintenance of animals

Adult animals were reproduced as described in our previous study to obtain embryos (Pompermaier et al., 2022). After the exposure period (3–120 hpf), the animals were kept in cell culture plates until day 7 to carry out all tests. After this period, they were transferred to aquariums with clean water for growth, where they were maintained at a density of 1 fish·L^{-1}. The environmental conditions were: an artificially regulated photoperiod (14 h of light and 10 h of dark), constant aeration, water temperature at 28 ± 2 °C, pH at 7.0 ± 0.2, dissolved oxygen at 6.2 ± 0.4 mg·L^{-1}, total ammonia at <0.01 mg/L, total hardness at 6 mg·L^{-1}, and alkalinity at 22 mg/L $CaCO_3$. The fish were fed with commercial flake food (42 % crude protein, 3400 kcal/kg) twice a day (08:00 and 17:00) and live food (*Artemia salina*) once a day. Partial water changes (25 %) were carried out thrice a week.

2.5. Persistent effect – behavioral testing

2.5.1. Novel tank test

At 180 days post-fertilization (dpf), the animals were evaluated for anxiety-like behavior using the novel tank test (NTT). This test was performed according to the methodology described by Pompermaier et al. (2021). Glass aquariums (24 × 8 × 20 cm, length × width × height) filled with chlorine-free water were used in this study. Fish were filmed for 6 min using a Logitech c920 HD webcam camera. The videos were analyzed using the automated tracking software ANY-maze® (Stoeling Co., Wood Dale, USA), in which the test tank was divided into the top, middle, and bottom zones. The behavioral parameters evaluated were distance traveled (m), absolute turn angle (°), entries into the top zone, time spent in the top zone (s), latency to enter the top zone (s), and time spent in the bottom zone (s). After filming each fish, the water was renewed. The number of animals filmed varied between 12 and 17 per group.

2.5.2. Aversivity test

To assess the impairment of the anti-predatory reactions of animals exposed to herbicides, we used an aversivity test to simulate the action of a predator. For the test, we used an aquarium measuring 12 × 12 × 5 cm containing 0.72 L of water (3 cm) and opaque walls virtually divided into a central zone and the periphery. The fish were acclimatized for 5 min, after which they received a predatory stimulus, consisting of mimicking the action of a predator with a mechanical apparatus. The behavior of the fish was recorded throughout the test period (5 min of acclimatization +5 min of exploration) using a Logitech® c920 HD webcam camera. The responses to the predation stimulus were analyzed using the automated tracking software ANY-maze® (Stoeling Co., Wood Dale, USA). The behavior of the animals before and after the stimulus was compared in terms of distance traveled (m), line crossings, entries into the central zone, time spent in the central zone (s), distance traveled in the central zone (m), and time spent in the peripheral zone (s). The number of animals filmed varied between 12 and 20 per group. This test was performed according to the methodology described by Pompermaier et al. (2020). After filming each fish, the water was renewed.

2.6. Reproduction and F1 larval parameters

F0 males and females aged 9–12 months were selected for breeding. They were placed in mesh-bottomed tanks in the afternoon to avoid egg predation. The following morning, embryos were collected by siphoning and washed to remove debris and feces. All eggs were counted and classified as fertilized or unfertilized. Fertilized eggs were used in the experiments. Embryos were maintained in 24-well cell culture plates (3 mL·well^{-1}), with 10 embryos per well, and incubated in a water bath at 28 °C for 7 days. Embryos maintenance conditions followed Pompermaier et al., 2022. Embryos up to 3 hpf were used (Kimmel et al., 1995). The process was repeated four times to obtain the number of embryos required for the control and glyphosate-treated groups. We attempted eight reproductions using the DBH-treated group but could not obtain any eggs.

2.6.1. Survival and hatching analysis

To obtain data on embryo and larval mortality, we monitored 180 individuals for seven days. To obtain hatching data, the embryos were monitored until 3 dpf, following the methodology described in our previous study (Pompermaier et al., 2022).

2.6.2. Spontaneous movement

At 24 hpf, the spontaneous movement of embryos within the egg was evaluated. The analysis was performed on 20 embryos per group, and the animals were monitored for 60 s using a stereomicroscope (Nova Instruments, Piracicaba, Brazil).

2.6.3. Heart rate

At 72 hpf, the animals' cardiac activity was evaluated. This parameter was chosen because the embryos have cardiac activity but little movement, which facilitates the evaluation. We monitored the heart rate for 60 s in 20 animals per group using a Nikon E-100 binocular biological microscope.

2.7. F1 generation behavioral testing

2.7.1. Open field test (OFT)

At 6 dpf the larvae were placed individually in 6-well cell culture plates with a total capacity of 15 mL, filled with 10 mL E3 medium, and filmed for 7 min (1 min acclimatization + 6 min exploration) (Pompermaier et al., 2022). To assess behavioral responses, we used 15–17 larvae per group. Larvae were filmed using the iPhone 6s iOS 14.6 smartphone camera function, and the videos were analyzed using the ANY-maze® automated tracking software (Stoelting Co., Wood Dale, USA). Because our objective was to evaluate thigmotaxic behavior, the test aquarium was virtually divided into a central zone and a periphery/wall zone. The parameters used to assess the behavior of the larvae were distance traveled (m), rotations, absolute turn angle (°), line crossings, entries into the central zone, time in the center zone (s), distance traveled in the center zone (m), time in the periphery zone (s), and distance traveled in the periphery zone (m). At the end of each test, the water in the well was changed.

2.7.2. Aversive stimulus test (AST)

The aversive stimulus test (AST) was chosen because it assesses the ability of larvae to identify possible threats from predators and move away from these risk zones. The test consisted of placing the larvae in 6-well cell culture plates with a total capacity of 15 mL and filled with 10 mL of E3 medium at 7 dpf, using 5 larvae per well with 15 replicates. The plates were placed on an LCD monitor, and, after 2 min of adaptation, we started exposure to the visual stimulus consisting of a 1.35 cm red sphere that covered only half of the well (Bridi et al., 2017; Pelkowski et al., 2011). The sphere's movement was performed using PowerPoint software (Microsoft Office Professional Plus 2016), and the larvae were exposed to the stimulus for 5 min. At the end of the test, the number of larvae remaining in the stimulus zone was evaluated (Kalichak et al., 2017). The larvae were filmed using an iPhone 6s iOS 14.6 smartphone camera. At the end of each test, the water in the well was changed.

2.8. Biochemical test

At 5 dpf, larvae were euthanized with ice water (Leary and Cartner, 2013), washed, and collected for biochemical analysis of acetylcholinesterase (AChE), catalase (CAT), and superoxide dismutase (SOD). Twenty larvae were used per pool, with four pools per group.

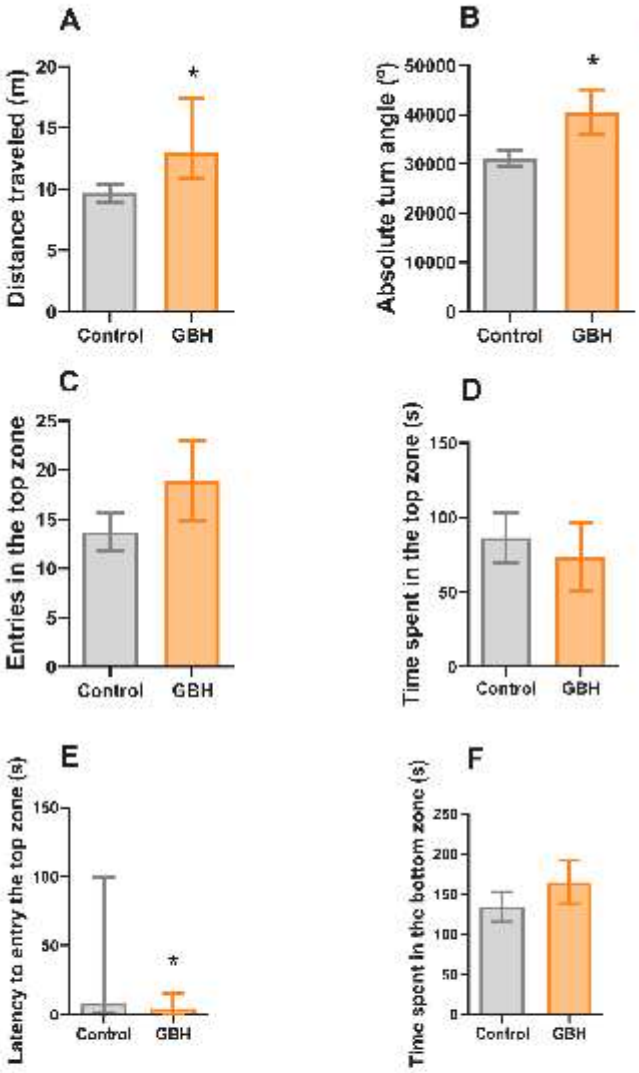

Fig. 2. Novel tank test results for adult fish exposed to glyphosate-based herbicides (GBH). (A) Distance traveled (m), (B) absolute turn angle (°), (C) entries into the top zone (s), (D) time spent in the top zone (s), (E) latency to enter the top zone (s), and (F) time spent in the middle zone (s). The data in panels B, C, D, E, and F, were compared using an unpaired *t*-test and are expressed as the mean ± SEM. The data in panel A were compared using a Mann-Whitney test and are expressed as median ± interquartile range (*$P < 0.05$) ($N = 12$–17).

2.8.1. Sample preparation

The whole-body tissue of twenty 5-dpf larvae was first homogenized in a Potter homogenizer for 1 min with 1 mL of Tris HCl buffer (50 mM, pH 7.4). They were then processed in an ultrasonic homogenizer in an ice bath for another 1 min. After that, the homogenate was centrifuged in Eppendorf tubes at 7000 × *g* for 10 min at 4 °C. The supernatant was collected for enzyme activity evaluation, and the pellet was discarded. The final homogenate was stored in an ultrafiltration freezer at −80 °C until analysis. The samples were analyzed in quadruplicate.

2.8.2. Protein determination

We followed the method described by Bradford (1976) to determine the protein concentration, using bovine serum albumin as the standard and Coomassie brilliant blue as the colorimetric reagent. The absorbance of each sample was measured at 595 nm and calculated using a standard curve. The concentration was expressed as mg·protein^{-1}.

2.8.3. Acetylcholinesterase (AChE) activity

The method described by Ellman et al. (1961) was used to determine AChE activity. The extract was prepared as described in Section 2.8.1. An ELISA microplate was pre-incubated for 5 min at 25 °C containing 15 μL of the sample, 115 μL of water (MilliQ), and 160 μL of Ellman's reagent (0.22 mM DTNB, dissolved in TFK buffer 11 mM, pH 7.5). The reaction was started by adding 30 μL of 8 mM ACh substrate, and the

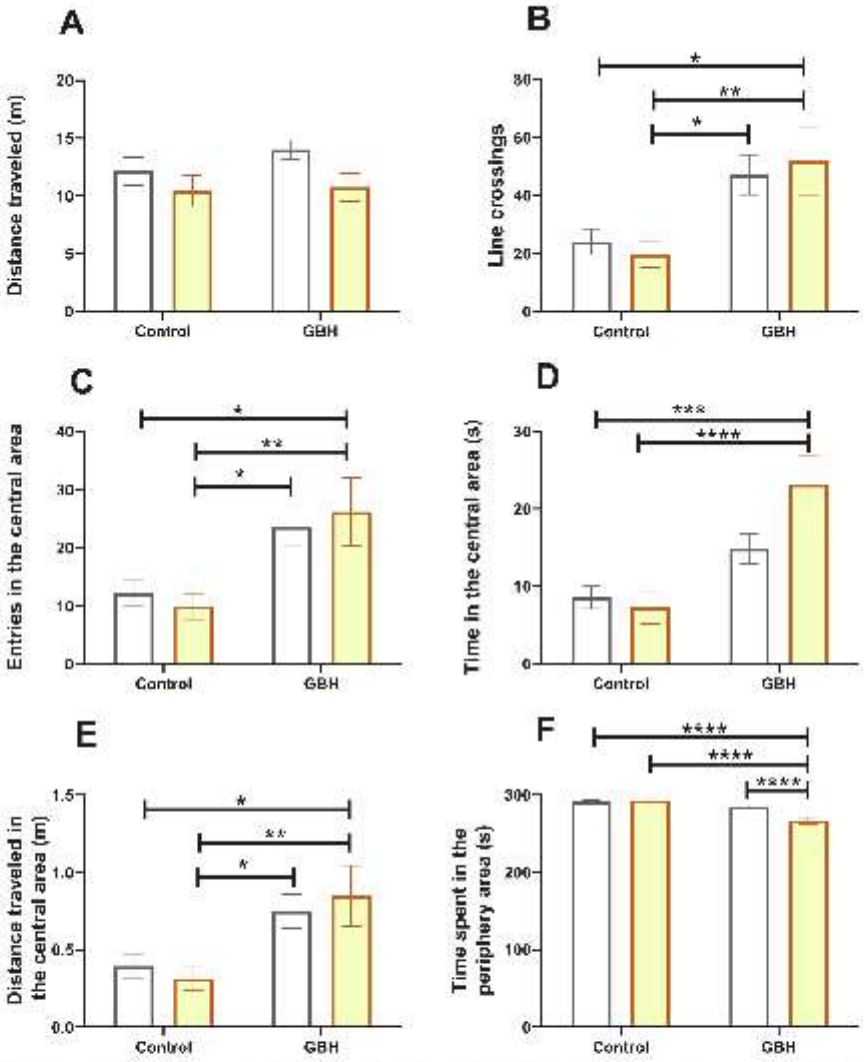

Fig. 3. Aversivity test results for adult fish exposed to glyphosate-based herbicides (GBH). (A) Distance traveled (m), (B) line crossings, (C) entries into the central zone, (D) time in the central zone (s), (E) Distance traveled in the central zone (m), and (F) time spent in the periphery zone (s). The white bars represent the values before the stimulus, while the orange bars the values after the predatory stimulus. Data are expressed by the mean ± SEM analyzed by two-way ANOVA with Tukey's post hoc test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) ($N = 12$–20).

absorption was measured at a wavelength of 405 nm for 5 min at 30 s intervals. Activity was expressed as μmol AChl protein/h/mg.

2.8.4. *Catalase (CAT) and superoxide dismutase (SOD) activity*

The same homogenate was used to assess CAT and SOD activities. CAT activity was measured according to Johansson and Borg (1988) and Goth (1991). The spectrophotometer was blanked with 10 μL of homogenate (20–30 μg of protein) in 1 mL of 50 mM potassium phosphate buffer (pH 7.5). Subsequently, 50 μL of hydrogen peroxide (0.3 M) was added. The reaction was quantified every 30 s for 2 min at a wavelength of 240 nm, and the activity was expressed U of CAT·mg protein^{-1}. The SOD activity was measured as described by Misra and Fridovich (1972) and Müller et al. (2018). Protein content was determined using the method established by Bradford (1976). For SOD activity determination, 50 μL of the sample (100 μg of protein) was added to 950 μL of glycine buffer (50 mM, pH 10.5). The enzymatic reaction was started by adding epinephrine (60 mM, pH 2.0). The colorimetric reaction was measured at 480 nm, and the activity was expressed as U of SOD/mg protein.

Table 1
Statistical discrimination of the results.

Parameter	Figure		F value	P value
Aversivity test				
Distance traveled	3A	Interaction	$F_{1,60} = 0.369$	0.5456
		GBH effect	$F_{1,60} = 0.693$	0.4084
		Stimulus effect	$F_{1,60} = 3.628$	0.0616
Line crossings	3B	Interaction	$F_{1,60} = 0.489$	0.4867
		GBH effect	$F_{1,60} = 17.35$	0.0001
		Stimulus effect	$F_{1,60} = 0.000$	0.9770
Entries into the central zone	3C	Interaction	$F_{1,60} = 0.499$	0.4824
		GBH effect	$F_{1,60} = 17.52$	<0.0001
		Stimulus effect	$F_{1,60} = 0.002$	0.9642
Time spent in the central zone	3D	Interaction	$F_{1,60} = 4.376$	0.0407
		GBH effect	$F_{1,60} = 23.55$	<0.0001
		Stimulus effect	$F_{1,60} = 2.308$	0.1340
Distance traveled in the central zone	3E	Interaction	$F_{1,60} = 0.6631$	0.4187
		GBH effect	$F_{1,60} = 16.45$	0.0001
		Stimulus effect	$F_{1,60} = 0.006$	0.9385
Time spent in the periphery zone	3F	Interaction	$F_{1,60} = 16.17$	0.0002
		GBH effect	$F_{1,60} = 43.57$	<0.0001
		Stimulus effect	$F_{1,60} = 12.15$	0.0009

2.9. Statistics

The NTT data were evaluated using the unpaired *t*-test or Mann-Whitney test for data not normally distributed by the Bartlett test. Aversivity data were analyzed using two-way ANOVA, followed by Tukey's test. Survival and hatching data were evaluated using the Kaplan-Meier method. For the analysis of data on heart rate, spontaneous movement, OFT, AST, and biochemical tests, the unpaired *t*-test or the Mann-Whitney test for data not normally distributed by the Bartlett test was used. All groups were compared with the control group. The significance level was set at 0.05. All statistical analyses were performed using GraphPad Prism® version 8.01 (GraphPad Software, San Diego, CA, USA). Data with normal distribution were represented as the mean ± SEM, while the non-parametric data as median ± interquartile range.

3. Results

3.1. Adult fish exposed as embryos or larvae (persistent effect)

3.1.1. GBH

3.1.1.1. Novel tank test (NTT). Fish exposed to GBH had a greater travel distance ($P = 0.0328$; $U = 54$; Fig. 2A), increased absolute turn angle ($P = 0.0382$; $t = 2.180$; Fig. 2B), and lower latency in the top zone ($P = 0.0431$; $t = 2.135$; Fig. 2E). There were no differences in the other parameters.

3.1.1.2. Aversivity test. There was no significant interaction between GBH exposure and stimuli. However, there was a significant effect in the line crossings where the fish exposed to GBH had a higher number of post-stimulus line crossings than that of the pre- and post-stimulus control group. Additionally, the number of line crossings was higher in the pre-stimulus GBH group than that for the post-stimulus control (Fig. 3B). There was no significant interaction between GBH exposure and stimuli, while there was a significant difference between the entries into the central zone where the post-stimulus GBH group entered the central zone more often than the pre- and post-stimulus control group. The pre-stimulus GBH group also entered the central zone more often than the post-stimulus control (Fig. 3C). There was a significant interaction between GBH exposure and time spent in the central zone. Fish exposed to GBH spent more time in this zone after the stimulus than before the stimulus or that of the pre-stimulus control group (Fig. 3D). There was no significant interaction of GBH exposure and stimuli, while there was a significant effect on the distance traveled in the central zone. Fish exposed to GBH traveled greater distances after the stimulus than the pre- and post-stimulus control group, and was also greater before the stimulus than in the post-stimulus control (Fig. 3E). There was an interaction and effect on the time spent in the periphery. Fish exposed to GBH spent less time than the pre- and post-stimulus controls, and post-stimulus GBH spent less time than pre-stimulus GBH (Fig. 3F). There was no difference in the total distance traveled (Fig. 3A). Statistical details were presented at Table 1.

3.2. DBH

3.2.1. NTT

DBH exposure did not affect adult fish. Statistical details were presented at Table 2.

3.2.2. Aversivity test

There was no significant interaction of DBH exposure and stimuli, while there was a significant effect on the time spent in the central zone, with the fish exposed to DBH spending more time in the central zone (Fig. 4D). There was no significant interaction of DBH and stimuli, while there was a significant effect in the periphery, where fish exposed to DBH spent less time in the periphery after the stimulus compared to the control before the stimulus (Fig. 4F). Statistical details were presented at Table 3.

3.3. Offspring evaluation

3.3.1. F1 embryonic parameters

Parental exposure to GBH during the embryonic and larval stages altered the survival rate ($x^2 = 6.037$; $P = 0.0140$; Fig. 5A) of F1 embryos and larvae. There were no changes in the other parameters.

3.3.2. Open-field test

F1 larvae of fish exposed to GBH showed a reduced total travel distance ($P = 0.0005$; $t = 3.874$; Fig. 6A), rotations ($P = 0.0237$; $t = 2.383$; Fig. 6B), absolute turn angle ($P = 0.0367$; $t = 2.187$; Fig. 6C), line crossings ($P = 0.0103$; $t = 2.736$; Fig. 6D), entries into the central zone

Table 2
Statistical discrimination of the results of novel tank test of fish exposed to DBH.

Parameters	Distance traveled (m)	Absolute turn angle (°)	Entries in the top zone	Time spent in the top zone (s)	Latency to entry the top zone (s)	Time spent in the bottom zone (s)
Control × DBH	$P = 0.1248$ $t = 1.579$	$P = 0.3337$ $t = 0.9826$	$P = 0.9915$ $t = 0.0106$	$P = 0.4382$ $t = 0.7856$	$P = 0.4624$ $t = 0.7456$	$P = 0.1217$ $t = 1.593$

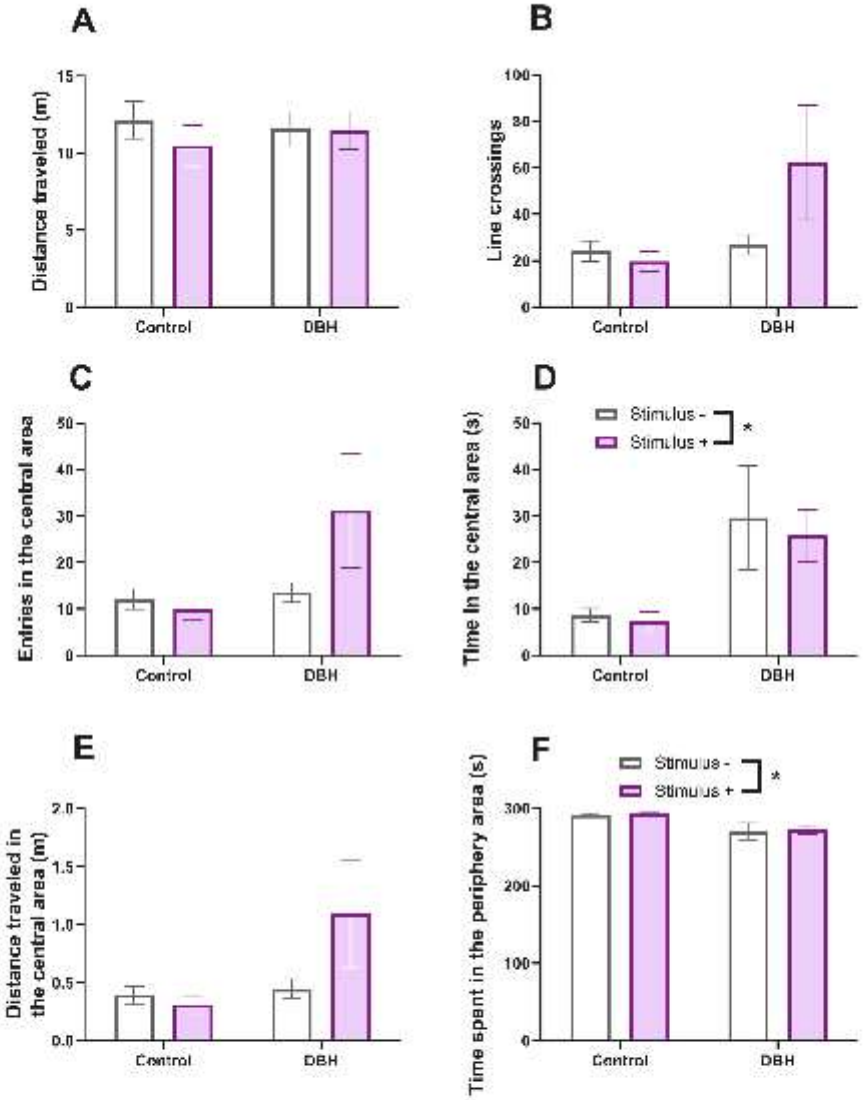

Fig. 4. Aversivity test results for adult fish exposed to 2,4-dichlorophenoxyacetic acid-based herbicides (DBH). (A) Distance traveled (m), (B) line crossings, (C) entries into the central zone, (D) time in the central zone (s), (E) Distance traveled in the central zone (m), and (F) time spent in the periphery zone (s). The white bars represent the values before the stimulus, while purple bars the values after the predatory stimulus. Data are expressed by the mean ± SEM analyzed by two-way ANOVA with Tukey's post hoc test (*$P < 0.05$) ($N = 12$–20).

($P = 0.0108$; $t = 2.720$; Fig. 6E), distance traveled in the central zone ($P = 0.0019$; $t = 3.407$; Fig. 6F), and distance traveled in the peripheral zone ($P = 0.0028$; $t = 3.251$; Fig. 6I). The time in the center ($P = 0.4272$; $t = 0.8049$; Fig. 6F) and in the periphery ($P = 0.7511$; $t = 0.3201$; Fig. 6H) did not differ between the groups.

3.3.3. *Aversive stimulus test*

There were fewer F1 larvae from fish exposed to GBH in the stimulated area after the predation simulation ($P = 0.0428$; $t = 2.119$; Fig. 7).

3.3.4. *Biochemical tests*

3.3.4.1. Acetylcholinesterase (AChE). F1 larvae from fish exposed to GBH had increased AChE activity; the same effect was observed in the

Table 3
Statistical discrimination of the results.

Parameter	Figure		F value	P value
Aversivity test				
Distance traveled	4A	Interaction	$F_{1,68} = 0.395$	0.5315
		DBH effect	$F_{1,68} = 0.025$	0.8740
		Stimulus effect	$F_{1,68} = 0.521$	0.4727
Line crossings	4B	Interaction	$F_{1,68} = 2.899$	0.0932
		DBH effect	$F_{1,68} = 3.764$	0.0565
		Stimulus effect	$F_{1,68} = 1.746$	0.1908
Entries in the central zone	4C	Interaction	$F_{1,68} = 2.884$	0.0940
		DBH effect	$F_{1,68} = 3.797$	0.0555
		Stimulus effect	$F_{1,68} = 1.746$	0.1908
Time in the central zone	4D	Interaction	$F_{1,68} = 0.047$	0.8276
		DBH effect	$F_{1,68} = 11.79$	0.0010
		Stimulus effect	$F_{1,68} = 0.199$	0.6567
Distance traveled in the central zone	4E	Interaction	$F_{1,68} = 2.803$	0.0987
		DBH effect	$F_{1,68} = 3.699$	0.0586
		Stimulus effect	$F_{1,68} = 1.696$	0.1972
Time spent in the periphery zone	4F	Interaction	$F_{1,68} = 0.002$	0.9589
		DBH effect	$F_{1,68} = 13.19$	0.0005
		Stimulus effect	$F_{1,68} = 0.079$	0.7793

parents ($P = 0.0009$; $t = 3.674$; Fig. 8).

3.3.4.2. Antioxidant enzymes. F1 larvae from fish exposed to GBH showed an increase in SOD activity ($P = 0.0009$; $U = 0$; Fig. 9A) and an inhibition of CAT activity ($P = 0.0387$; $U = 73$; Fig. 9B).

4. Discussion

In our previous study, exposure to GBH affected survival, behavior, and AChE activity during the embryonic stage, whereas exposure to DBH affected behavior and AChE (Pompermaier et al., 2022). In this study, we showed that fish exposed to GBH in their embryonic and larval stages showed hypermobility, and their anti-predatory reaction was impaired during adulthood, indicating a persistent effect. We also showed that fish had impaired survival and behavioral changes as well as affected AChE and antioxidant enzyme activity in the F1 generation, characterizing a transgenerational effect. In fish exposed to DBH, the adult fish could not reproduce despite the effects verified in the embryos (Pompermaier et al., 2022) not persisting into adulthood, demonstrating that the exposure affected the perpetuation of the species.

Adult fish exposed to GBH showed hypermobility and lower latency for entry to the top in the NTT test. The expected behavior in the NTT test is that the fish initially stay at the bottom of the aquarium and, after a few moments, begin to explore the new environment (Kysil et al., 2017). However, the lower latency for entry into the top zone showed that the GBH-exposed fish were anxious and explored the environment quickly after starting the test. This anxiolytic-like behavioral pattern leaves the fish susceptible to predation, as it facilitates detection by the predator and can decrease the chances of survival.

GBH exposure also impaired their ability to react to the predator since they stayed longer, entered, and swam more in the central zone in the aversivity test. These reactions demonstrate that the fish did not recognize the risk and could not react to the predatory stimulus. The same behavior has already been observed in larvae in an aversive stimulus test (AST) (Pompermaier et al., 2022) and adult fish acutely exposed to GBH in an aversivity test (Pompermaier et al., 2020). Anti-predatory reactions are critical for ensuring species survival and maintaining an ecological balance (Kelley and Magurran, 2003; Stewart et al., 2013). Situations where the predator has an advantage can result in an imbalance in the ecosystem, which can affect survival and even lead to extinction.

These effects observed for GBH-exposed fish in the NTT and aversivity tests clearly indicate that the effects observed in early life were persistent. Fish that were exposed as embryos and larvae carried the effects of exposure because, as adults, they showed the same behavioral pattern as in their initial developmental stage.

Epigenetic alterations can explain these persistent effects. Our exposure window comprised the entire period of animal organogenesis (3–120 hpf), during which fish are sensitive to epigenetic disruptions caused by chemicals (Gao et al., 2018). As epigenetic processes regulate embryonic development (Feng et al., 2010; Groh et al., 2015; Morgan et al., 2007), the effects of changes during this specific period can also be observed in adulthood, demonstrating a persistent effect (Groh et al., 2015). We did not evaluate possible DNA methylation in larvae and adults; however, this does not preclude the epigenetic-centered hypothesis.

The effects observed in GBH-exposed fish can also be explained by the mechanisms of action already described in the literature, such as alterations in AChE (Pompermaier et al., 2022), disruption of the hypothalamic-pituitary-interrenal axis (Cericato et al., 2008, 2009; Koakoski et al., 2014), and oxidative stress (de Moura et al., 2017; Faria et al., 2021; Liu et al., 2022). GBH has been reported to cause behavioral and morphological changes at different stages of zebrafish development, in addition to a significant impairment in long-term memory in the inhibitory prevention test (Bridi et al., 2017).

In the DBH-exposed fish, no changes were observed in their behavior when they reached the adult stage; however, the initial exposure to DBH caused mild hypermobility and increased AChE activity in the larvae (Pompermaier et al., 2022). Because the concentration was relatively low (3.4 $\mu g \cdot L^{-1}$), we cannot discard the possibility that the exposed fish may not have shown an effect. Other studies have demonstrated the effect of this compound, both on behavior and physiology, at higher concentrations. This includes impairment of anti-predatory activity in fish exposed to 29 $\mu L \cdot L^{-1}$ (Pompermaier et al., 2020), inhibition of AChE with exposure to 10 $mg \cdot L^{-1}$ (da Fonseca et al., 2006), increased CAT activity, resulting in a reduced SOD/CAT ratio, at concentrations of 30 and 300 $\mu g \cdot L^{-1}$ (Thiel et al., 2020), cardiotoxicity at 25 and 50 $mg \cdot L^{-1}$ and oxidative stress at 25 $mg \cdot L^{-1}$ (Li et al., 2017).

However, fish exposed to DBH were unable to reproduce, which may impair the perpetuation of the species and cause a significant change in the ecological balance. This infertility observed in DBH-exposed fish can be explained by possible effects on oocytes, as already demonstrated in other studies using DBH, where fish showed a decrease in the number of oocytes, deformed and underdeveloped oocytes, and an increase in the number of atretic oocytes (Koç and Akbulut, 2012). This may demonstrate that DBH causes a delay in fish oogenesis. In addition, exposure to DBH caused a decrease in fecundity (Coady et al., 2013).

In addition, exposure to other contaminants also impaired their reproduction ability. Initial exposure to bisphenol A (BPA) or 17α-ethinylestradiol (EE2) compromised the reproduction of the F2 generation, showing a greater effect of exposure over generations (Bhandari et al., 2015). Fish exposed to benzo(a)pyrene (BaP) had fewer eggs, a lower fertilization rate, and decreased hatching success (Gao et al., 2018). Indeed, some endocrine-disrupting chemicals (EDCs) are increasingly associated with reproductive dysfunction, male-to-female sex ratio shifts, reduced fertility, reproductive tract abnormalities, and

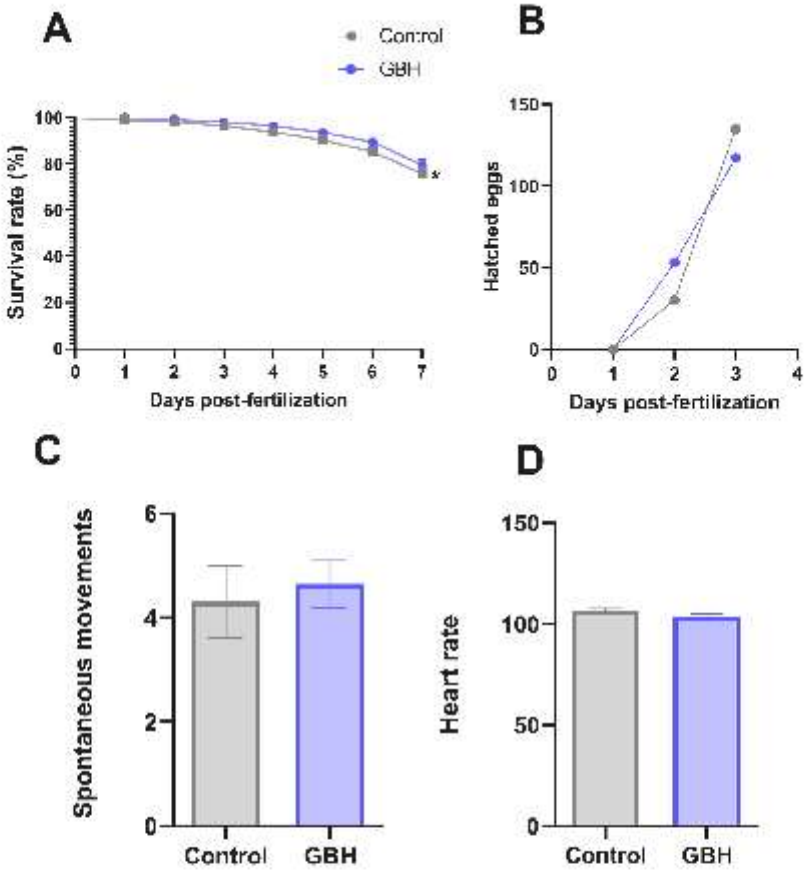

Fig. 5. Developmental parameters of F1 zebrafish larvae exposed to glyphosate-based herbicides (GBH). (A) Survival rate (%) ($N = 180$), (B) hatched eggs ($N = 180$), (C) spontaneous movements ($N = 20$), and (D) heart rate (s) ($N = 20$). Survival rates and hatched eggs were compared using Kaplan-Meier survival curves. Spontaneous movements were compared using the unpaired *t*-test and are expressed as mean ± SEM (*$P < 0.05$).

precocious puberty in fish (Ghanem, 2021). Therefore, the previously reported effects of DBH exposure on fish may be the reason for the lack of eggs in several reproduction trials.

Regarding transgenerational effects, early exposure of embryos and larvae to GBH impaired the survival of F0 larvae, as reported in our previous study (Pompermaier et al., 2022). The same effect was observed in larvae of the F1 generation, which also had changes in their survival curve. Based on this, we showed that exposure to GBH causes damage to the perpetuation of the species, as it reduces the number of individuals over generations. In addition, some studies have demonstrated the effects of GBH exposure on other early developmental parameters, such as mortality (Webster et al., 2014), premature hatching (Liu et al., 2022), reduced heart rate (Gaur and Bhargava, 2019; Liu et al., 2022), pericardial and yolk sac edema, deficiency of the swim bladder, and a shortened body length (Liu et al., 2022).

Regarding the transgenerational transmission of behavioral effects, we observed significant changes in the F1 generation, where the animals were calmer and less anxious as they traveled shorter distances and moved away from the central and risk zones, both in OFT and AST. This effect is the opposite of that observed in F0 larvae and adult fish, which were hyperactive and had impaired anti-predatory reactions. This pattern of behavior demonstrated by F1 larvae with decreased locomotion and exploration is harmful to the species, as an undamaged behavior pattern is crucial for the survival of fish (Stewart et al., 2013). Therefore, the effects observed in F1 larvae represent a risk for the species, as the animals may have difficulty finding mates and also feeding, and they might be more susceptible to predation.

The larvae of the F1 generation of fish exposed to GBH were also evaluated for biochemical biomarkers because organophosphates can change the activity of acetylcholinesterase (AChE). We verified increased AChE activity, which may explain the behaviors observed in larvae of the F1 generation. AChE plays a fundamental role by acting on muscle junctions and in the synaptic clefts of cholinergic neurons, where it catalyzes the neurotransmitter acetylcholine (ACh) into acetate and choline. When a reduction in its activity occurs, there is excitability of the cholinergic system due to the increased permanence of ACh in the synaptic cleft. In contrast, increased AChE activity reduces the permanence of ACh in the synaptic cleft, thereby reducing the efficiency of

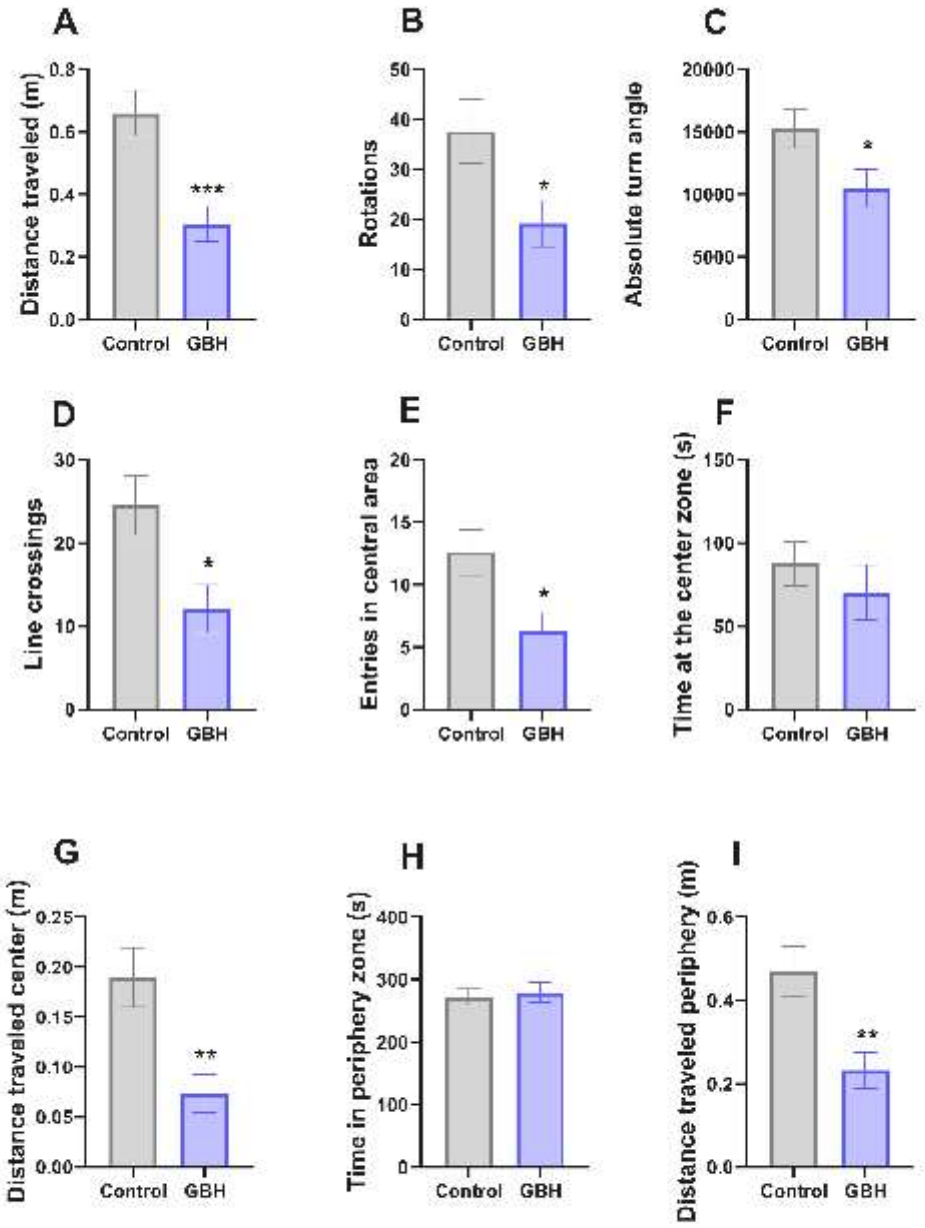

Fig. 6. Open-field test results for F1 larvae exposed to glyphosate-based herbicides (GBH). (A) Distance traveled (m), (B) rotations, (C) absolute turn angle (°), (D) line crossings, (E) entries into the central zone (s), (F) time in the center zone (s), (G) distance traveled center (m), (H) time in the periphery zone (s) and (I) distance traveled in the periphery zone (m). The data in the panels were compared using an unpaired *t*-test and are expressed as mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) ($N = 15$–17).

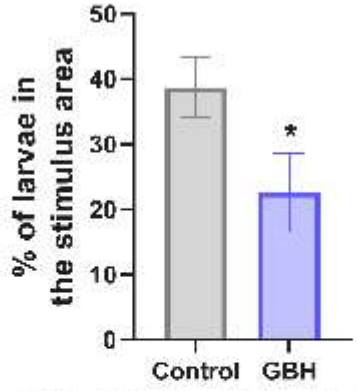

Fig. 7. Aversive stimulus test in F1 fish exposed to glyphosate-based herbicides (GBH). The data were compared using an unpaired *t*-test and are expressed as mean ± SEM ($^{*}P < 0.05$) ($N = 75$).

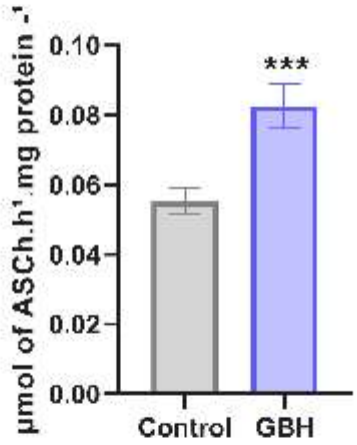

Fig. 8. Acetylcholinesterase in F1 fish exposed to glyphosate-based herbicides (GBH). The data were compared using an unpaired *t*-test and are expressed as mean ± SEM ($^{***}P < 0.001$) ($N = 16$).

synapses, which can result in changes in behavior, attention, cognition, and natural brain responses. Therefore, the effects observed can be explained by increased AChE activity, which made the animals calmer and less anxious.

As for the first line of antioxidant defense (SOD and CAT), the larvae of the F1 generation, unlike F0 larvae, showed an increase in SOD activity and inhibition of CAT activity. As a first-line defense against oxidative damage, the enzymes SOD and CAT (Ighodaro and Akinloye, 2018) play key roles in the metabolism of reactive oxygen species (ROS) that are harmful to the organism, transforming them into less toxic compounds that the body can excrete. Changes in the levels of these enzymes demonstrate that the antioxidant system can prevent oxidative damage. Here, we showed that the activity of both enzymes was altered in the F1 generation. The increase in SOD levels may indicate a possible mechanism for GBH-induced oxidative stress. GBH interacts with cell membranes and increases lipid damage. This interaction with cellular structures leads to mitochondrial dysfunction and increases intracellular superoxide ion levels (Ranjan and Jindal, 2022). Many organophosphates are linked to bioaccumulation due to their high affinity for lipophilic tissues and might constantly react to dysfunction of natural cellular metabolism. SOD dismutates the superoxide ion and increases the concentration of hydrogen peroxide, another reactive but less toxic species. Therefore, we can hypothesize that GBH accumulates in the larval tissue and causes this process. Additionally, due to the large production of free radicals that increase SOD activity, there is an exacerbated presence of H2O2 in cells, which inhibits CAT activity (Aguilar et al., 2020). As there was low sample size, we obtained wide confidence intervals in the antioxidant enzymes determinations, which creates uncertainty regarding these results. Therefore, caution is recommended when interpreting our SOD and CAT results. However, this possible limitation does not lessen the global relevance of our work, since data about persistent and transgenerational effects of GBH and DBH are scarce.

The changes in biochemical biomarkers observed in the F1 generation larvae may be the key to the behavioral effects observed in both OFT and AST. The increased AChE associated with oxidative stress can lead to cell apoptosis and death in animals. This series of results clearly demonstrate the transgenerational effect of GBH exposure. The question as to how these are carried to future generations remains.

The transgenerational transmission of behavioral and biochemical changes may also be explained by epigenetics, reinforcing our epigenetic-based explanation of the persistent effects. A clear limitation of our work is that we did not evaluate DNA methylation in larvae and adults. However, this does not lessen the merit and relevance of our work since little is known about the effects of pesticides on different generations of fish, especially regarding persistent and transgenerational effects on behavioral parameters.

Altogether, our results suggest a serious impact caused by the initial exposure of fish embryos and larvae to environmental concentrations of pesticides, which can compromise the entire life of the animal and subsequent generations. To this end, we recall the 17 sustainable development goals (SDGs) of the United Nations (UN) that aim to ensure a balanced environment for future generations, setting the guarantee of safe drinking water and sanitation (SDG 6) and conservation of life in water (SDG 14) as specific goals. Therefore, the impacts of water contamination shown in this study must be considered in decision-making regarding the use and regulation of pesticides so that these impacts can be curbed and more sustainable solutions that preserve life and aquatic ecosystems can be reached.

Ethics approval and consent to participate

This study was approved by the Ethics Commission for Animal Use Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #0016/2019 - CEUA).

Consent for publication

Not applicable.

CRediT authorship contribution statement

A.P.: Conceptualization, Experimental work, Writing - Original draft preparation.

W.A.T.: Experimental work and laboratory analyses.

C.A.: Experimental work and laboratory analyses.

L.J.G.B.: Conceptualization, Supervision Data analysis, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial

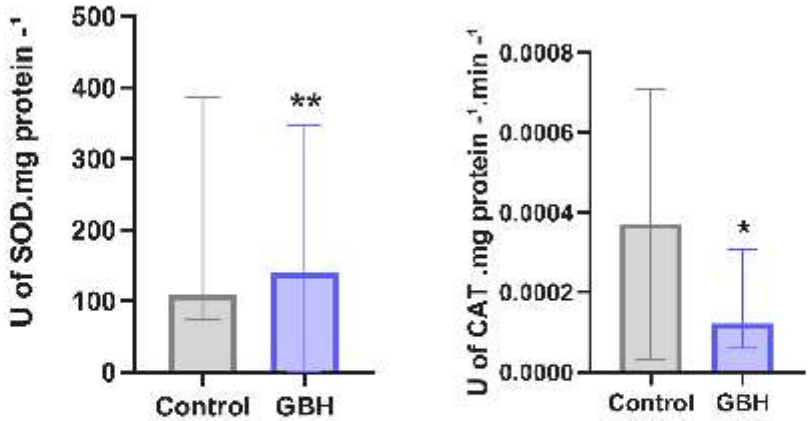

Fig. 9. Antioxidant enzymes in F1 fish exposed to glyphosate-based herbicides (GBH). The data in the panels A and B were compared using a Mann-Whitney test and are expressed as median ± interquartile range (*$P < 0.05$, **$P < 0.01$) ($N = 5$–16).

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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5. CONCLUSÕES

Aqui mostramos que a exposição ao glifosato afetou a geração F0 através da diminuição da sobrevivência, causou hipermobilidade e comportamento ansiolítico, afetou negativamente o comportamento antipredatório das larvas e aumentou a atividade da acetilcolinesterase. Na fase adulta, os peixes expostos ao glifosato apresentaram hipermobilidade e sua reação antipredatória foi prejudicada, caracterizando um efeito persistente. As larvas da F1, dos peixes expostos ao glifosato, tiveram alterações na sobrevivência e no comportamento, bem como efeitos na atividade da AChE e enzimas antioxidantes, caracterizando um efeito transgeracional.

A exposição ao 2,4-D causou ligeira hipermobilidade nas larvas da geração F0 e aumento da atividade da acetilcolinesterase. Na fase adulta, os peixes não apresentaram persistência dos efeitos observados na fase embrionária e larval, porém, eles não foram capazes de se reproduzir, o que demonstra que a exposição afetou a perpetuação da espécie.

Essas alterações observadas nos animais expostos ao glifosato e ao 2,4-D (Quadro 2) onde foi possível observar efeito em todas as fases dos animais podem comprometer a perpetuação da espécie, a busca por parceiros/alimentos e facilitar a ação de predadores, o que pode resultar em graves desequilíbrios ecológicos e comprometer a sobrevivência da espécie.

Quadro 2: Efeitos da exposição ao glifosato e 2,4-D em peixes-zebra

Substância	**Fase da vida**	**Efeitos**
Glifosato	**Embrionária e larval**	Diminuição da sobrevivência, hipermobilidade, comportamento ansiolítico, prejuízo no comportamento anti-predatório e aumento da AChE
	Adulta	Hipermobilidade e reação anti-predatória prejudicada.
	Embrionária e larval – F1	Prejuízo na sobrevivência, alterações comportamentais, aumento da AChE e SOD e inibição da AChE
2,4-D	**Embrionária e larval**	Ligeira hipermobilidade e aumento da AChE
	Adulta	Não reproduziram

6. CONSIDERAÇÕES FINAIS

A contaminação da água por resíduos de agrotóxicos é uma realidade e os efeitos nos organismos não-alvo vem sendo investigados cada dia mais. De acordo com os resultados demonstrados nessa tese de doutorado podemos evidenciar que uma exposição na janela inicial da vida animal desencadeia uma série de efeitos ao longo da sua vida, com prejuízos na sua sobrevivência, mudanças no seu comportamento e alterações fisiológicas, o que pode comprometer toda a sua sobrevivência e a perpetuação da espécie.

Em conjunto nossos resultados acendem um alerta sobre as consequências dessas contaminações, pois mesmo um derrame acidental pode impactar toda a vida do animal e até mesmo as suas próximas gerações e isso pode acontecer sem que seja notada a presença desse composto no corpo d'água ou em concentrações que são permitidas pela lei.

Por mais que medidas mitigatórias sejam adotadas para que se controle a disposição e presença desses compostos na água os efeitos das contaminações poderão ser observados por longos períodos. Por isso é de fundamental importância que soluções mais sustentáveis sejam pensadas e propostas para podermos atingir a produção das monoculturas sem causar tanto impacto para o ambiente e para as espécies não-alvo.

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ANEXOS

UNIVERSIDADE DE PASSO FUNDO
VICE-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

CERTIFICADO

Certificamos que a proposta intitulada **"EFEITOS PERSISTENTES E TRANSGERACIONAIS DOS RESÍDUOS DE AGROTÓXICOS"**, registrada com o **nº 016/2019** sob a responsabilidade de **Leonardo José Gil Barcellos** e que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos) para fins de Pesquisa, encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **aprovada** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE DE PASSO FUNDO (CEUA-UPF) em reunião de 21/10/2020.

Finalidade: Pesquisa

Espécie/linhagem/raça: Danio Rerio (Peixe zebra)

Peso/idade: 0,5g *Nº de animais: 1200*

Sexo: Machos e Fêmeas

Origem: Laboratório de Fisiologia de Peixes da FAMV

Resumo: Os agrotóxicos chegam aos corpos hídricos de diversas formas, seja pelo descarte incorreto de embalagens, aplicação direta para controle de macrófitas, lixiviação das lavouras ou pelos processos naturais de degradação. No ambiente Aquático espécies não alvo acabam por entrar em contato com essas substâncias ficando susceptíveis a efeitos adversos ainda pouco elucidados. O peixe-zebra apresenta muitas vantagens como embriões translúcidos, rápido desenvolvimento e custos mais baixos de manutenção e por isso, vem sendo utilizado largamente para estudo dos efeitos dos poluentes emergentes. Com o objetivo de avaliar os efeitos persistentes e transgeracionais da contaminação por diferentes agrotóxicos sobre as relações interespecíficas em peixe-zebra, os experimentos serão realizados no Laboratório de Fisiologia de Peixes do Hospital Veterinário, da Faculdade de Agronomia e Medicina Veterinária (FAMV), na Universidade de Passo Fundo (UPF), campus Passo Fundo. O primeiro experimento se baseará na exposição crônica dos embriões aos herbicidas glifosato e 2,4-D, e acompanhamento do seu desenvolvimento inicial com análises dos padrões de mortalidade e eclosão, movimentação espontânea, frequência cardíaca e mudanças no comportamento exploratório e anti predatório. A segunda etapa do projeto consiste em analisar o efeito persistente sofrido por esses animais expostos. Os peixes, quando adultos, serão submetidos aos testes do tanque novo e do estímulo predatório, onde será avaliado se o efeito da contaminação persiste na fase adulta dos animais expostos quando embriões. Estes mesmos animais, após a maturação sexual, serão reproduzidos para dar origem a F1, onde serão realizados os mesmos testes da F0, para verificar se os animais demonstram efeitos transgeracionais. A hipótese a ser testada pelo presente projeto é que os principais agrotóxicos utilizados na classe dos herbicidas, prejudicam o desenvolvimento inicial do peixe-zebra e tem efeitos que são persistentes e transgeracionais.

Passo Fundo, 26 de outubro de 2020.

Prof. Dr. Rafael Frandoloso
Coordenador CEUA /UPF