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Jaíne Ames

EFEITOS DO GLIFOSATO SOBRE O DESENVOLVIMENTO EMBRIONÁRIO, LARVAL E ADULTO DO PEIXE-ZEBRA (*Danio rerio*)

SANTA MARIA, RS
2021

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

Orientadora: Prof. Drª. Vania Lucia Loro

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Como é possível que seres inteligentes tenham almejado controlar umas poucas espécies indesejadas por um método que contaminou todo o meio ambiente e trouxe a ameaça da doença e da morte inclusive para sua própria espécie? Precisamos urgentemente acabar com essas falsas garantias, com o adoçamento as amargas verdades. A população precisa decidir se deseja continuar no caminho atual, e só poderá fazê-lo quando estiver em plena posse dos fatos.

Rachel Louise Carson

RESUMO

EFEITOS DO GLIFOSATO SOBRE O DESENVOLVIMENTO EMBRIONÁRIO, LARVAL E ADULTO DO PEIXE-ZEBRA (*Danio rerio*)

AUTORA: JAÍNE AMES
ORIENTADORA: VANIA LUCIA LORO

Os Herbicidas à Base de Glifosato (HBG) são os agrotóxicos mais utilizados no mundo e representam riscos à saúde ambiental e a de organismos não-alvos. Esse trabalho teve como objetivo verificar a toxicidade, os mecanismos envolvidos e as consequências da exposição a HBG em peixe-zebra (*Danio rerio*). Para isso, desenvolvemos dois capítulos independentes, sendo um experimental e outro uma revisão sistemática com meta-análise. No primeiro, analisamos a toxicidade de um HBG em concentrações permitidas pela legislação na água potável ao longo do desenvolvimento embrionário e larval do peixe-zebra. Embriões de peixe-zebra foram expostos às concentrações permitidas de glifosato em água potável em embriões de peixe-zebra, sendo a metade (250 µg/L), dosagem máxima permitida (500µg/L) e o dobro (1000 µg/L) de glifosato Roundup Original DI®. Mortalidade, taxa de eclosão, frequência cardíaca, malformações, comportamento e marcadores bioquímicos foram avaliados. Nossos resultados apontam um aumento na mortalidade em 6 horas pós fertilização (hpf) na concentração de 500 µg/L, aumento na taxa de eclosão e na frequência cardíaca a 48 hpf em todas as concentrações e aumento no número de animais com malformações em todas as concentrações. Em relação aos marcadores bioquímicos, encontramos uma diminuição na atividade das enzimas AChE e GST. No segundo, estudamos os mecanismos envolvidos na toxicidade do glifosato na fase embrionária, larval e adulta do peixe-zebra através de um procedimento meta-analítico. Para isso, foi realizada uma busca nas bases de dados PubMed e Science Direct. Artigos avaliando o efeito do glifosato em peixes-zebra publicados até julho de 2020 foram considerados. Após a obtenção dos dados, o software R foi utilizado para a realização dos testes meta-analíticos. A seleção por título, resumo e texto completo foi realizada, e 20 artigos originais de pesquisa com peixes-zebra expostos ao glifosato comercial e/ou puro foram obtidos. O estudo de revisão sistemática e meta-análise mostrou que o glifosato induz aumento da mortalidade, causa malformações, afeta a eclosão e a frequência cardíaca nas fases embrionária e larval. Em altas concentrações, encontramos uma maior probabilidade de observar um efeito na morfologia, eclosão, malformações e mortalidade. Além disso, observamos danos bioquímicos e comportamentais causados na fase adulta. Em conjunto, nossos dados sugerem danos no desenvolvimento embrionário, larval e adulto do peixe-zebra, o que sugere um prejuízo à saúde dos peixes e de outros organismos aquáticos expostos a formulações contendo glifosato.

Palavras-chave: Herbicida. Peixe-zebra. Embrião. Larva. Adulto. Ecotoxicologia.

ABSTRACT

EFFECTS OF GLYPHOSATE ON EMBRYO, LARVAL AND ADULT DEVELOPMENT OF ZEBRAFISH (*Danio rerio*)

AUTHOR: JAÍNE AMES
ADVISOR: VANIA LUCIA LORO

Glyphosate-Based Herbicides (GBH) are the most used pesticides in the world and pose risks to environmental health and to non-target organisms. This work aimed to verify the toxicity, the mechanisms involved and the consequences of exposure to GBH in zebrafish (*Danio rerio*). For this, we developed two independent chapters, one experimental and the other a systematic review with meta-analysis. In the first, we analyzed the toxicity of an GBH in concentrations allowed by legislation in drinking water throughout the embryonic and larval development of zebrafish. Zebrafish embryos were exposed to the permitted concentrations of glyphosate in drinking water in zebrafish embryos, half (250 µg/L), maximum (500µg/L) it's the double (1000 µg/L) of permitted concentrations in drinking water of glyphosate Roundup Original DI®. Mortality, hatch rate, heart rate, malformations, behavior and biochemical markers were evaluated. Our results point to an increase in mortality within 6 hours after fertilization (hpf) at a concentration of 500 µg/L, an increase in hatching rate and heart rate at 48 hpf at all concentrations. An increase in the number of animals with malformations was recorded in all concentrations tested. Regarding biochemical markers, we found a decrease in the activity of AChE and GST enzymes. In the second, we study the mechanisms involved in glyphosate toxicity in the embryonic, larval and adult stages of zebrafish through a meta-analytic procedure. For this, a search was performed in the PubMed and Science Direct databases. Articles evaluating the effect of glyphosate on zebrafish published up to July 2020 were considered. After obtaining the data, the R software was used to perform the meta-analytical tests. Selection by title, abstract and full text was performed, and 20 original research articles with zebrafish exposed to commercial and/or pure glyphosate were obtained. The systematic review and meta-analysis study showed that glyphosate induces increased mortality, causes malformations, affects hatching and heart rate in the embryonic and larval stages. At high concentrations, we found a higher probability of observing an effect on morphology, hatching, malformations and mortality. In addition, we observed biochemical and behavioral damage caused in adult phase. Taken together, our data suggest damage to the embryonic, larval and adult development of zebrafish, which suggests harm to the health of fish and other aquatic organisms exposed to formulations containing glyphosate.

Keywords: Herbicide. Zebrafish. Embryo. Larval. Adult. Ecotoxicology.

LISTA DE ABREVIATURAS E SIGLAS

AChE - Acetilcolinesterase

HBG - Herbicida à Base de Glifosato, do inglês, G*lyphosate-Based Herbicide*

CAT - Catalase

GST - Glutationa S-Transferase

ACAP - Capacidade Antioxidante Contra Peróxidos, do inglês, *Antioxidant Capacity Against Peroxyl Radicals*

I.A. - Ingrediente Ativo

ROS - Espécies Reativas ao Oxigênio, do inglês, *Reactive Oxygen Species*

TBARS - Substâncias Reativas ao Ácido Tiobarbitúrico, do inglês, *Thiobarbituric Acid-Reactive Substance*

NPSH - Tióis Não Proteicos, do inglês, *Non-Protein Thiols*

PE - Edema Pericárdico, do inglês, *Pericardial Edema*

YBE - Edema no Saco Vitelino, do inglês, *Yolk Bag Edema*

BM - Malformações no Corpo, do inglês, *Body Malformations*

SC - Curvatura Espinhal, do inglês, *Spinal Curvature*

HPF - Horas Pós Fertilização

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1 APRESENTAÇÃO

A presente dissertação aborda uma problemática ambiental extremamente relevante e com necessidade urgente de mais evidências sobre os prejuízos causados por agroquímicos ao ambiente e aos seres vivos que nele habitam. Desta forma, esta dissertação está estruturada da seguinte forma. Primeiro, apresento uma Introdução geral sobre a temática abordada, incluindo uma breve revisão de literatura e os objetivos. Para contemplar os objetivos gerais e específicos desta dissertação, foram realizados estudos separados que estão apresentados em dois capítulos específicos nominados "Herbicida à Base de Glifosato (HBG) induz danos no desenvolvimento de embriões de peixe-zebra (*Danio rerio*)" e "Efeitos do glifosato no peixe-zebra (*Danio rerio*): uma revisão sistemática e meta-análise". Um estudo experimental avaliou os efeitos da exposição de herbicida a base de glifosato a dosagens presentes na água potável em embriões de peixe-zebra; e uma revisão sistemática e meta-análise sobre os efeitos da exposição ao glifosato no ciclo de vida do peixe-zebra. As metodologias, resultados e discussão estão disponíveis nas seções específicas de cada manuscrito. A sessão Discussão Geral apresenta de forma integrada os resultados obtidos nessa dissertação. Por fim, a Conclusão expõe aspectos gerais resultantes dos estudos conduzidos.

2 INTRODUÇÃO

A água é um recurso natural e fundamental para a vida de todos os organismos da Terra. Pois, através de suas características físico-químicas permitem o funcionamento do metabolismo bioquímico. A importância da qualidade da água está bem conceituada na Política Nacional de Recursos Hídricos, que define, dentre seus objetivos, "assegurar à atual e às futuras gerações a necessária disponibilidade de água, em padrões de qualidade adequados aos respectivos usos" (BRASIL, 1997). Porém, a água é afetada pela contaminação e poluição de efluentes industriais, esgotos domésticos, domissanitários, resíduos agrícolas, que inevitavelmente alcançam os recursos hídricos (AMIARD-TRIQUET et al., 2015).

Os mecanismos de poluição das águas podem ser divididos em duas categorias, que são as fontes pontuais e fontes difusas (não pontuais). A primeira pode ser identificada e diagnosticada, e ela se trata do descarte de esgotos domésticos ou industriais, tratados ou não. Ao contrário das fontes pontuais, as fontes difusas são aquelas cuja origem não pode ser facilmente identificada, podendo ser transportadas de inúmeras maneiras até atingir o corpo aquático receptor (MERTEN e MINELLA, 2002). As fontes difusas se tratam da deposição atmosférica úmida e seca (WU et al., 1992), a lixiviação de compostos do solo, drenagem de águas pluviais (MITCHELL, 2005) e a partir de atividades agrícolas (ZAGATTO e BERTOLETTI, 2008). A contaminação por atividades agrícolas pode ser por meio da aplicação no solo de fertilizantes e pesticidas (GONÇALVES et al., 2000). Atualmente, os pesticidas são considerados um dos tipos de poluentes emergentes, ou seja, não são frequentemente monitorados, porém apresentam o potencial de causar efeitos adversos nos ambientes aquáticos bem como à saúde humana (GEISSEN et al., 2015). A agricultura aprimorou-se de modo a ser mais produtiva, potencializando e intensificando a produtividade agrícola, sendo necessária a aplicação de pesticidas (SILVA e FAY, 2004). Nesse sentido ocorreu um aumento de 589% no uso de pesticidas em todo o mundo, de acordo com dados de 1990-2010 (FAO, 2020 a,b,c).

Dentre os pesticidas mais utilizados atualmente no Brasil e no mundo, os herbicidas à base de glifosato (GBHs, do inglês *Glyphosate-based herbicides*), estão liderando a lista como os mais produzidos e usados em áreas agrícolas em todo o mundo (BENBROOK, 2016). Estes herbicidas são utilizados para remover ervas daninhas indesejadas, mais frequentemente usado em áreas agrícolas em todo o mundo (MYERS et al., 2016). Ele foi descoberto em 1950 por um químico suíço que trabalhava em uma empresa farmacêutica e se chamava Dr. Henri Martin (DILL et al., 2010). Em 1970 foi identificado com atividade herbicida pelo Dr. John Franz, químico da Monsanto e em 1974 teve sua formulação realizada e vendido pela Monsanto,

chamando-se Roundup (DUKE e POWLES, 2008). Esse herbicida pertence à classe toxicológica IV, apresentando toxicidade moderada (ver nota técnica nº 23/2018 da ANVISA, a qual recomenda que o glifosato seja reclassificado como Classe I, extremamente tóxico). O glifosato nas plantas inibe a atividade da 5-enolpiruvilshiquimato-3-fosfato sintase (EPSPs), uma enzima chave da biossíntese de aminoácidos aromáticos, e prejudica os processos metabólicos gerais, como a síntese de proteínas e fotossíntese (FAO, 2016).

Em uma escala global, foram utilizados até o ano de 2014 cerca de 800 milhões de kg de glifosato, sendo utilizados para fins agrícolas (90%) e fins não agrícolas (10%) (BENBROOK, 2016). Já no Brasil, de acordo com o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA 2021), no ano de 2019 foi comercializado o total de 620.537,98 toneladas de ingrediente ativo e é o mais vendido. Devido a essa ampla gama de utilização dos GBHs, existem diferentes formulações no mercado, com a combinação do ingrediente ativo glifosato e outros aditivos (surfactantes e surfactantes) (LANCTÔT et al., 2014). Além disso, as moléculas de glifosato sofrem a ação dos microrganismos, que os decompõem em metabólitos, como o ácido aminometilfosfônico (AMPA), que é altamente persistente no ambiente (SVIRIDOV et al., 2015; OKADA et al., 2020).

Estes dados apresentados evidenciam a massiva utilização, produção e comercialização desse herbicida na agricultura, podendo contaminar os ecossistemas aquáticos. Nesse ambiente eles podem entrar por meio de pulverização, aplicações diretas de pulverização excessiva, por escoamento, lixiviação, pelo descarte inadequado de embalagens, pelo vento, gerando sérias preocupações (SILVA e FAY, 2004; ABRANTES et al., 2009; VILLAMAR-AYALA et al., 2019; CARLES et al., 2019). Também, a ampla gama de aplicações desse herbicida (antes da semeadura e secagem pré-colheita) e sua meia-vida que pode variar de 1 a 91 dias na água, levam à sua presença prevalente em ecossistemas aquáticos (CARVALHO, 2006; ANNETT et al., 2014; HÉBERT et al., 2019; POHL et al., 2019).

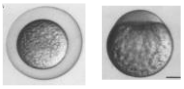
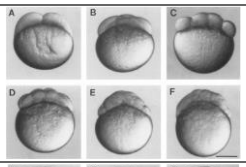
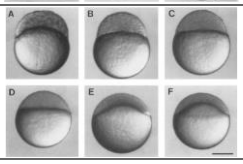
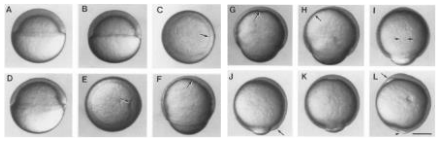
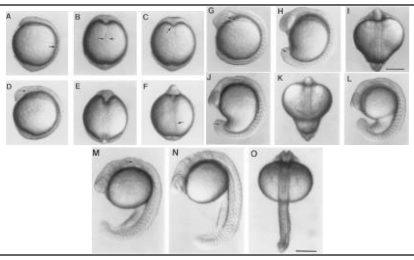
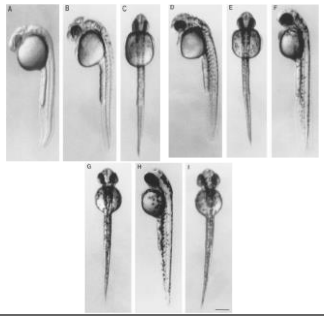
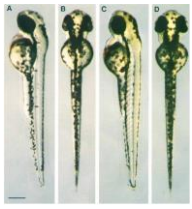
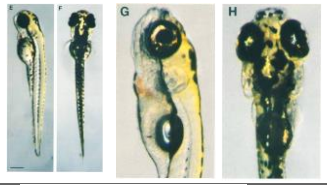
Assim, o glifosato tem sido registrado em rios, riachos, lagos, lagoas e pântanos nos Estados Unidos (BATTAGLIN et al., 2014); águas superficiais na Argentina (APARICIO et al., 2013); águas superficiais na Europa (POIGER et al., 2017); águas superficiais no Brasil (FERNANDES et al., 2019); rios e córregos no Canadá (GLOZIER et al., 2012) Esses dados evidenciam a sua persistência no ambiente aquático e levantam preocupações sobre potenciais ameaças ambientais e de saúde pública (HONG et al., 2018). Existem legislações que controlam os níveis seguros de alguns pesticidas em água para consumo humano e dessedentação de animais. Segundo a Portaria GM/MS nº 888, de 4 de maio de 2021 o valor máximo permitido

para o glifosato na água potável para consumo humano é de 500 µg/L (BRASIL, 2021). Os valores-limite permitidos para o glifosato na água potável variam entre os países. A concentração máxima permitida na União Europeia é 0,1 µg/L, na Austrália é 1000 µg/L e no Canadá é 280 µg/L (EC, 1998; Australian Drinking Water Guidelines, 2011; Guidelines for Canadian Drinking Water Quality, 2019).

Devido a essa problemática, embora o mecanismo de ação-alvo do glifosato seja específico para plantas, vários estudos têm demonstrado efeitos deletérios do glifosato em espécies de peixes, variando de genotoxicidade (MORENO et al., 2014), aumento do estresse oxidativo (DE MOURA et al., 2017), alterações morfológicas (GLUSCZAK et al., 2007), danos ao fígado (TOPAL et al., 2015), desenvolvimento embrionário anormal (ZHANG et al., 2017), imunotoxicidade (MA et al., 2015) e alteração do perfil metabólico (LI et al., 2017). Esses efeitos podem ser causados em peixes, uma vez que eles possuem a capacidade de absorver e concentrar toxinas diretamente da água circundante ou indiretamente de outros organismos (pequenos peixes, invertebrados e vegetação aquática) (POLAT et al., 2016). Além disso, eles estão submetidos a diferentes formulações, como já citado anteriormente.

Os peixes são amplamente utilizados em ensaios de toxicologia ambiental. Além disso, vantagens como: ampla distribuição (ocorrendo em grande variedade de hábitats), diversidade de espécies, fácil identificação e contribuem para avaliações toxicológicas através de variadas metodologias (GRABARKIEWICZ e DAVIS, 2008). Um dos organismos com grande potencial de aplicação para estudar efeitos de pesticidas na água, é o peixe-zebra (*Danio rerio*, Hamilton 1822). É uma espécie da família Cyprinidae, originário do sudeste da Ásia (Bangladesh, Índia, Nepal, Tailândia), e caracterizado por apresentar listras azuis e brancas ao longo do corpo e da nadadeira anal e caudal (ENGESZER et al., 2007). É encontrado em seu habitat natural em cardumes de 5 a 20 indivíduos (LAWRENCE, 2007). Este peixe é usado como espécie bioindicadora da contaminação aquática, pois possui desenvolvimento rápido, possuem de 3 a 4 centímetros, alta fecundidade, elevado grau similaridade genética (70%) ao ser humano e atende ao critério dos 3 Rs (GRUNWALD e EISEN, 2002; HOWE et al.,2013). O estágio de desenvolvimento do peixe-zebra é organizado nos seguintes períodos: zigoto, clivagem, blástula, gástrula, segmentação, faríngula, eclosão, larval, juvenil e adulto (ver tabela adaptada de KIMMEL et al., 1995). A utilização do peixe-zebra nos seus diferentes estágios de desenvolvimento, permitem a utilização de diferentes metodologias com diferentes avaliações. O que permite avaliar os prejuízos causados pelo glifosato durante toda a sua vida, obtendo respostas que se completam.

Tabela 1. Estágios de desenvolvimento do peixe-zebra (*Danio rerio*).

Período	Tempo	Morfologia	Desenvolvimento
Zigoto	0 - 0.75 h		Ovo recém-fertilizado. O citoplasma flui em direção ao pólo animal.
Clivagem	0.75 - 2.25 h		Após a primeira clivagem, as células (blastômeros) se dividem em intervalos de 15 min. As seis clivagens ocorrem em orientações regulares.
Blástula	2.25 - 5.25 h		O embrião entra na transição da blástula média, a camada sincicial da gema se forma e a epibolia se inicia.
Gástrula	5.25 - 10.33 h		A epibolia continua, ocorrem os movimentos celulares morfogenéticos, produzindo as camadas germinativas primárias e o eixo embrionário.
Segmentação	10.33 - 24 h		Movimentos morfogenéticos ocorrem, os somitos se desenvolvem, os órgãos primários tornam-se visíveis, a cauda torna-se mais proeminente e o embrião se alonga.
Faríngula	24 - 48 h		O embrião é bilateralmente organizado e a notocorda bem desenvolvida. O cérebro é esculpido em 5 lobos. A cabeça se endireita. As barbatanas começam a se formar. As células pigmentares se diferenciam. O sistema circulatório se forma e o coração começa a bater. Ocorre desenvolvimento comportamental.
Eclosão	48 - 72 h		A morfogênese de muitos órgãos agora está completa, com algumas exceções, incluindo o intestino e seus órgãos associados. Pode-se observar o desenvolvimento das barbatanas peitorais, das mandíbulas e das guelras.
Larva	72 h - 29 d		A boca está bem aberta e se projeta logo após o olho. A lâmina da barbatana peitoral continua a se expandir. A melanina se acumula. O tom amarelo de todo o aspecto dorsal do corpo aumentou.
Juvenil	30 - 89 d		Comprimento total do corpo é de 14 mm e 12 dentes.
Adulto	90 d - 2 anos		Adulto reprodutor

Considerando o contexto apresentado, devido ao uso massivo do glifosato na agricultura e o risco real de contaminação aquática por esse herbicida, é crucial avaliar seu potencial risco sobre os peixes. Nesse sentido, a ideia desta dissertação é demonstrar os mecanismos de toxicidade do glifosato em toda a vida do peixe-zebra (desde a embrionária até a adulta). A partir de um estudo experimental considerando os efeitos do glifosato em concentrações presentes na água potável na fase embrionária e larval do peixe-zebra. E uma revisão sistemática com meta-análise que aborda os efeitos causados pelo glifosato durante todas as fases da vida do peixe-zebra.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar os efeitos e os mecanismos envolvidos na toxicidade mediada pela exposição ao glifosato em peixe-zebra (*Danio rerio*) durante as fases embrionária, larval e adulta.

3.2 OBJETIVOS ESPECÍFICOS

Os objetivos específicos estão apresentados em cada estudo que compõe esta dissertação.

3.2.1 Herbicida à Base de Glifosato (HBG) induz danos no desenvolvimento de embriões de peixe-zebra (*Danio rerio*)

- Avaliar se o GBH em concentrações presentes na água potável afeta a mortalidade, taxa de eclosão, frequência cardíaca, malformações e comportamento de embriões;
- Verificar se o GBH em concentrações presentes na água potável altera os parâmetros antioxidantes enzimáticos, não enzimáticos, peroxidação lipídica e danos oxidativos.

3.2.2 Efeitos do glifosato no peixe-zebra (*Danio rerio*): uma revisão sistemática e meta-análise

- Investigar os efeitos do glifosato em todas as fases de vida do peixe-zebra;
- Avaliar possíveis rotas envolvidas na toxicidade do glifosato no peixe-zebra.

4 CAPÍTULO 1: HERBICIDA À BASE DE GLIFOSATO (HBG) INDUZ DANOS NO DESENVOLVIMENTO DE EMBRIÕES DE PEIXE-ZEBRA (*Danio rerio*)

Este capítulo apresenta os danos causados pelo herbicida à base de glifosato Roundup Original DI® nas fases embrionária e larval do peixe-zebra. Este estudo gerou o seguinte artigo que foi submetido para a publicação na revista "*Aquatic Toxicology*".

GLYPHOSATE BASED HERBICIDE (GBH) INDUCE DAMAGES IN THE DEVELOPMENT OF ZEBRAFISH EMBRYOS (*Danio rerio)*

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Abstract: Glyphosate-based herbicides (GBH) are the most widely used pesticides in the world. Glyphosate, pure or mixed with surfactants (commercial formulation) can induce damage in the development, morphology, and reproduction of non-target organisms. Our work aimed to evaluate the effects of *Danio rerio* embryos exposed to glyphosate, especially the doses allowed for human consumption in drinking water. Zebrafish embryos were exposed to 250, 500, and 1000 µg/L of Roundup Original DI® glyphosate for 96 hours. Mortality, hatching rate, heart rate, malformations, behavior, and biochemical markers were evaluated. Our results showed that at 6 hpf the animals exposed to the concentration of 500 µg/L had an increase in mortality compared to the control. Regarding the hatching rate, it was observed that it increased in all groups exposed to the control at 48 hpf. In our study, the embryos exposed to glyphosate did not present changes in the spontaneous movement and touch response. We observed that the groups exposed had a higher number of animals with malformations compared to the control. The malformations found were pericardial edema, yolk sac edema, body malformations, and curvature of the spine. Regarding changes in heart rate, we can see that tachycardia occurred in all groups exposed to GBH, as predicted due to cardiac abnormalities. We found a significant decrease in GST enzyme activity in the GBH 250 and GBH 500, and a significant decrease in AChE enzyme in the GBH 250. No differences were found between the groups about the concentration of protein, ACAP, TBARS, ROS, NPSH, and catalase. The damage in all evaluated natural stages of development was is aggravated by the mortality rate and systemic and morphological changes. The large-scale use of glyphosate, coupled with the permissiveness to its presence in daily human food, causes irreparable damage to the aquatic environment, as it affects non-target animals.

Keywords: drinking water pollution; embryo malformation; metabolic damage; mortality.

1. Introduction

Freshwater is an important natural and fundamental resource for human health and the survival of organisms. However, with human population growth and the accelerated industrialization, urbanization, and agricultural practices, threats to the health of freshwater environments are growing (Reid et al., 2019). Residues of industrial, agricultural, and sewage can reach the freshwaters and they can lead to water pollution. Pollution from agricultural activities is increasingly present, due to the increasing use of pesticides in agriculture practices (Amaral et al., 2020). Pollutants cause changes in water quality, which can lead to the destruction of biodiversity and changes in the entire ecosystem. Furthermore, causing various diseases to humans and a shortage of drinking water. About more than 50% of water pollution in streams and rivers occurs due to leaching and mixing of chemicals from agricultural practice (US EPA, 2014).

One of the most popular pesticides is glyphosate [N- (phosphonomethyl) glycine], a broad-spectrum systemic herbicide and crop desiccant controlling broadleaf weeds and grasses (Benbrook, 2016; Duke, 2017). Nearly 800 million kg of glyphosate were used worldwide for

agricultural purposes (90%) and non-agricultural purposes (10%) (Benbrook, 2016). Glyphosate has been widely used in Brazil, for example, according to the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA 2019), glyphosate is the active ingredient most sold in Brazil (217 tonnes). The major concern of the excessive use of glyphosate is because many commercial formulations have surfactants as additives sometimes being more toxic than the pesticide itself (Lanctôt et al., 2014; Murussi et al., 2016). The massive use of this herbicide and its additives in agriculture demonstrates its constant presence in rivers and drinking water (Cengiz et al., 2017; Fernandes et al., 2019).

Brazil presents some guidelines indicating the safe levels of some pesticides in the water for human and animal consumption. According to Brazil (2017), the maximum allowed value for glyphosate in drinking water for human consumption is 500 µg/L (acceptable intake). The permitted limit values for glyphosate in drinking water vary across countries. The maximum permitted concentration in European Union is 0.1 µg/L, in Australia is 1000 µg/L, and in Canada is 280 µg/L (EC, 1998; Australian Drinking Water Guidelines, 2011; Guidelines for Canadian Drinking Water Quality, 2019). Furthermore, the maximum concentration of glyphosate in drinking water suggested by the EPA (900 µg/L) is based on the usage amounts, toxicological data, environmental residual levels, and acceptable daily intake (ADI, 0.3 mg kg^{-1} day^{-1}) (Baer and Marcel, 2014). However, some studies evaluated the glyphosate concentration allowed by the drinking water legislation can cause negative effects on aquatic organisms (Faria et al., 2021; Fiorino, 2018; Smith et al., 2019). Moreover, empirical studies evaluating change in embryonic development are still scarce (Bridi et al., 2017).

Zebrafish (*Danio rerio*, Hamilton 1822) are very important in exposure to toxic substances in the embryonic process (Li et al., 2020; Shen et al., 2020; Sun et al., 2020; Wang et al., 2020; Zhang et al., 2020). In this period of its cycle of life, the most important transformations occur when all the structures of the body are defined (Kimmel et al., 1995). Therefore, it is interesting to evaluate the exposure of toxic compounds, permanent changes in the development of the organism, and subsequently its health and even behavior or survival. The use of the zebrafish embryo to assess the toxicity of compounds has advantages, such as (1) transparent chorion, allowing to observe its development; (2) production of thousands of embryos daily; (3) low maintenance cost; (4) rapid development; (5) small-scale analysis and an alternative for correlation with in vivo tests with adult fish (Lammer et al., 2009; Scholz et al., 2008). Early zebrafish assays are particularly suitable for chemical evaluation since the early stages of development are very sensitive to compounds (Braunbeck et al., 2015).

Considering the massive use of glyphosate in world agriculture, and the real risk of aquatic contamination by this herbicide even at permitted concentrations for drinking water, it is necessary to evaluate the possible effects on non-target organisms, such as the zebrafish. Therefore, this study aimed to investigate the effects of commercial glyphosate (Roundup Original DI®) on embryo development, biochemical markers, and behavior of zebrafish. Although there are studies on glyphosate toxicity in embryos zebrafish (Bridi et al., 2017; Gaur and Bhargava, 2019; Lanzarin et al., 2020; Moraes et al., 2020), no study has used this glyphosate formulation and concentrations permitted by law for human consumption in drinking water during the early life stages.

2. Materials and methods

2.1 Zebrafish maintenance and reproduction

Wild-type adult zebrafish (*Danio rerio*; WT) matrices used to obtain the embryos were kept under a photoperiod of 14 h light/10 h dark and appropriate water conditions (dechlorinated, aerated, temperature 27±1 ºC, and pH 7.2±0.5). These animals were purchased from a local supplier in Santa Maria, Rio Grande do Sul, Brazil. The matrices used were kept in an aquatic toxicology laboratory, fed twice a day (9 am and 3 pm) with commercial Ovo-vit ration (Tropical®), and supplemented with *Artemia* sp. For breeding, 36 animals (24 males and 12 females) of zebrafish were used, kept in 30L aquariums, each aquarium containing 2 females and 4 males. The zebrafish embryos used in the present work were obtained from the matrices reproduction and kept in a room with a temperature of 27ºC ±1 and a photoperiod of 14h light/10h dark. After breeding the zebrafish, embryos were collected, immersed in E3 medium (containing 5mM NaCl, 0.17mM KCl, 0.33mM CaCl, 0.33mM $MgSO_4$, and 1% blue methylene) and placed on a petri dish for random selection, according to Westerfield (2000). The experimental protocols were approved by the local Animal Ethics Committee of the Federal University of Santa Maria, under the number: 6192220819.

2.2 Experimental exposure

The exposure protocol used for glyphosate assays was adapted from Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). After random selection, the embryos (no abnormality in cell division or chorion) were placed in 24-well plates (kept at 27ºC ±1) with a total of 5

embryos per well, and the wells were filled with 2 mL of the water containing glyphosate or not (control) within 3 hours post-fertilization (hpf). After that, in 6 hpf a mortality test was performed; 24 hpf mortality and hatching; 28 hpf spontaneous movement; 48 hpf mortality, hatching, and heart rate; 72 hpf mortality, hatching, touch response, and malformations; and 96 hpf mortality, hatching and biochemical analyzes. The glyphosate solution was prepared using commercial formulation Glyphosate Roundup Original DI® (Di-ammonium salt 445g/L; Monsanto do Brasil Ltda.) diluted in dechlorinated water. The experimental groups were: (1) control (0, without glyphosate); (2) GBH 250 (250 µg/L of Glyphosate Roundup Original DI®); (3) GBH 500 (500 µg/L of Glyphosate Roundup Original DI®); (4) GBH 1000 (1000 µg/L of Glyphosate Roundup Original DI®). The value of 500 µg/L is due to the maximum value permitted for Brazilian environment law (Brasil, 2017). One hundred and twenty embryos (120, 24 wells X 5 embryos) were exposed per group (0, 250, 500, or 1000 µg/L) and the experimental protocol was made in duplicate.

2.3 Mortality and hatching rate

Mortality was evaluated at 6, 24, 48, 72, and 96 hpf time points. We chose to assess mortality at 6 hpf because cell division occurs at this stage (Kimmel et al., 1995). Embryos viability was analyzed by a blinded observer through the presence of coagulation of eggs and the absence of the heartbeat in embryos and larvae. The hatching rate was analyzed by a blinded observer who counted the animals that hatched at 24, 48, 72, and 96 hpf. All parameters described here were assessed according to previously published protocols and Fish Embryo Acute Toxicity Test (OECD 2013; Westerfield 2000) and adapted according to Severo et al. (2020).

2.4 Heart rate

The animals with 48 hpf were selected randomly and placed in Petri dishes and they were observed under a Professional Digital Microscope 300x Zoom USB coupled Vastar Mega Pixels 500X microscope. For the heart rate analysis, 30 animals from each group were filmed for 1 minute by a blind observer and the number of heartbeats observed in that time interval was counted (adapted from (Xia et al., 2017).

2.5 Spontaneous movement of body axes

Embryos with 28 hpf were randomly selected from a total of 15 embryos (in duplicate) from each group for the analysis of spontaneous movement. In this period, the first movement made by the embryo occurs, which originates from the spinal cord triggered by the development of motor neurons that innervate the muscles (Saint-Amant and Drapeau, 1998; Xia et al., 2017). These embryos were placed individually in Petri dishes containing treatment water and were able to adapt for 2 min. After that, with the aid of a magnifying glass, a blind observer counted the movements performed for 1 minute (Costa-Silva et al., 2018).

2.6 Touch response

Embryos hatched at 72 hpf were submitted to touch response test analysis (adapted from (Saint-Amant and Drapeau, 1998). This test is important to detect the individual's reaction to the stimulus since in the absence of stimulus the animals remain immobile. Briefly, one embryo at a time randomly selected was gently placed in the center of a Petri dish containing 20 mL of treatment solution. After the 1-minute interval for ambiance, a needle stimulus was performed by a blind observer, and the number of stimuli necessary for the first displacement to occur was counted of the larva towards the plate edge was counted. This behavior was assessed by two blind operators (30 embryos per group).

2.7 Malformations

Larvae with 72 hpf were randomly selected, and the number of animals with the presence and absence of malformations was counted. These morphological changes were Pericardial Edema (PE), Yolk Bag Edema (YBE), Body Malformations (BM) (including tail malformation, short tail, and head malformation), and Spinal Curvature (SC). Subsequently, the animals were photographed with the aid of a Professional Digital Microscope 300x Zoom USB coupled Vastar Mega Pixels 500X microscope (Sulukan et al., 2017).

2.8 Biochemical markers

After 96 hpf exposure, 30 zebrafish larvae were pooled and placed in a plastic microtube and sacrificed under 60 min of hypothermia. A total of 6 micro-tubes were used for each treatment. After water removal, 200 µL of 50 mM Tris-HCl buffer (pH 7.4) were added and the

samples homogenized with a glass cane, and centrifuged (3000 g for 10 min, - 4 °C). The supernatant was transferred to microtubes and kept at −80 °C for subsequent assays. Protein was determined in all tissues based on the methodology of Bradford (1976), expressed as mg of protein/mL. Catalase (CAT) activity was expressed as μmol/min/mg of protein, according to Nelson and Kiesow (1972). Glutathione S-transferase (GST) activity was analyzed according to Habig et al. (1974) and expressed as μmol GS-DNB/min/mg of protein. Acetylcholinesterase (AChE) activity was determined according to Ellman et al. (1961) and expressed as μmol/min/mg of protein. To evaluate Reactive Oxygen Species (ROS) and total antioxidant capacity against peroxyl radicals (ACAP), we used the methods of Ali et al. (1992) and Amado et al. (2009), respectively. ROS was expressed in pmol DFC/mg of protein and ACAP as the relative area of ROS. Lipid peroxidation levels were determined by thiobarbituric acid-reactive substance (TBARS) production, expressed in nmol MDA/mg of protein by the method of Draper and Hadley (1990). To determine non-protein thiols (NPSH) levels we used the method described by Ellman (1959), and results were expressed in nanomole SH/milligram of protein.

2.9 Statistical analysis

The data were tested for normality using the Kolmogorov-Smirnov test and homogeneity of variances using the Bartlett test. To investigate whether the mortality and hatching parameters differed significantly between groups according to time, a two-way ANOVA analysis of variance was performed, followed by a Tukey post-hoc test. To assess whether malformations and heart rate differed between groups, a one-way ANOVA test followed by a Tukey post-hoc test was performed. The behavioral tests were statistically analyzed by the Kruskal-Wallis test and Dunn's Multiple Comparison Test. To investigate whether the biochemical parameters differ significantly between groups, a one-way ANOVA was performed, followed by a Tukey post-hoc test. The data were expressed as mean values ± standard error (SEM). Differences with $p < 0.05$ were considered significant. Statistical analyzes were performed using the GraphPad Prism 6.0 program for Windows (GraphPad Software, San Diego, USA).

3. Results

3.1 Mortality and hatching

We investigated the effect of Glyphosate Roundup Original DI® exposure on mortality and hatching at 6, 24, 48, 72, and 96 h after the beginning of the exposure. Those zebrafish in normal formation with cell divisions or tissue differentiation were considered to be alive. The mortality results at 6 hpf demonstrated that animals exposed to a concentration of 500 µg/L had an increase in mortality about the control ($p<0.0001$). Considering the other concentrations (250 and 1000 µg/L), the mortality was lower than the values found at 500 µg/L of glyphosate (Figure 1A). Regarding the hatching rate, was observed that it increased in all groups exposed to glyphosate compared to the control at 48 hpf ($p<0.05$; Figure 1B).

3.2 Malformations

When analyzing the malformations at 72 hpf, we observed that the groups exposed to glyphosate had a higher number of animals with malformations compared to the control ($p<0.05$; Figure 2). We also found that the group exposed to 500 µg/L had the largest number of animals with malformations ($p<0.05$; Figure 2). These malformations were pericardial edema, yolk sac edema, body malformations (including tail malformation, short tail, and head malformation), and spinal curvature (Figure 3).

3.3 Touch response and Spontaneous movement

The animals were submitted to a stimulus through touch, which was used as a measure of sensorimotor integration at 48 hpf. There was no significant difference between groups exposed to glyphosate. The embryos responded to the touch of the tail with wave contractions of the body axis and capable of swimming and they did not differ from control ($p>0.05$; Figure 4A). Animals exposed to glyphosate did not present changes in the spontaneous movement evaluated at 28 hpf ($p>0.05$; Figure 4B).

3.4 Heart rate

Embryo exposure to all concentrations of glyphosate-based herbicide resulted in a change in heart rate. These alterations were verified by the increase in the number of heartbeats per minute compared to the control group ($p <0.05$; Figure 5).

3.5 Biochemical analysis

The exposure of zebrafish embryos to glyphosate caused a decrease in the GST activity in groups 250 µg/L ($p = 0.0035$) and 500 µg/L ($p = 0.0065$) when compared to the control (Figure 6E). The activity of the enzyme acetylcholinesterase was significantly decreased in group 250 µg/L ($p = 0.0387$) when compared to the control (Figure 6F). No significant differences were found between the groups in relation to other biochemical markers analyzed, including concentration of protein ($F_{(3, 28)} = 0.08326$, $p= 0.9686$), ACAP ($F_{(3, 28)} = 2.913$, $p= 0.0518$), TBARS ($F_{(3, 27)} = 1.371$, $p= 0.2729$), ROS ($F_{(3, 26)} = 0.3751$, $p= 0.7717$), NPSH ($F_{(3, 20)} = 1.274$, $p= 0.3104$) and the activity of the enzyme catalase ($F_{(3,12)} = 0.08228$, $p= 0.9684$) (Figure 6).

4. Discussion

The indiscriminate use of glyphosate in crops is a worldwide problem since this herbicide can be carried to nearby aquatic environments in large quantities and periodically causing negative effects on non-target organisms, such as adult fish (Battaglin et al., 2014; Moreno et al., 2014; Murussi et al., 2016; Shiogiri et al., 2012). Considering that glyphosate is capable of causing effects in adult fish, it is essential to evaluate its toxicity in one of the most critical phases of the animal, the embryo-larval phase. All the implications of the pollutants at this stage may affect the life and survival of the fish. Thus, it is to be expected that in the embryonic phase these animals are more susceptible than adults to pesticides (Lammer et al., 2009). The discrepancy in sensitivity between zebrafish embryos and adult animals can be explained by the lack of fully developed metabolic pathways to degrade pesticides (Embry et al., 2010). Many researchers have carried out the exposure of zebrafish embryos to glyphosate (Gaur and Bhargava, 2019; Lanzarin et al., 2020; Moraes et al., 2020; Roy et al., 2016), however, the effects of glyphosate in concentrations stipulated by regulatory agencies have not generally been evaluated according to toxicity studies. In Brazil, the maximum acceptable value of glyphosate in surface waters for human consumption is defined as 500 µg/L (Brazil, 2017). However, checking the potential risks of the concentrations present in drinking water to the health of organisms at different phylogenetic levels should be better explored. If these concentrations then affect different organisms, it could pose a threat to public health.

In this study, we observed that glyphosate in all tested concentrations affected the development in the embryo-larval phase of the zebrafish (*Danio rerio*). This herbicide affected hatching rate, survival, heart rate and caused embryonic and larval deformities. Regarding the

mortality rate, we observed that the largest number of dead embryos in all groups occurred within six hours post fertilization (6 hpf). This is because at this stage cell division occurs, and this is one of the most sensitive moments for embryo development (Kimmel et al., 1995). We observed that the first 6 hours in contact with glyphosate (500 µg/L) affected the survival of the embryos, with the highest number of deaths. In other periods analyzed (24, 48, 72 e 96 hpf) we found that glyphosate did not affect embryo survival at the concentrations used. Similarly, our results to use of concentrations of commercial glyphosate at 0.01 and 0.5 mg/L (10 e 500 µg/L) found no change in the survival rate in 24 hpf (Uren Webster et al., 2014).

Hatching is one of the important stages in the fish life cycle, which directly affects the rate of embryo development (Yumnamcha et al., 2015). The normal time for embryos to hatch is 48 to 72 hours after fertilization, depending on enzyme activity and embryonic movements (Kimmel et al., 1995; Samaee et al., 2015). In our results, there was an increase in the number of hatches in 48hpf in the groups exposed to glyphosate compared to the control group. If hatching occurs ahead of time, the embryo may become more susceptible to dangerous situations in the environment (Kimmel et al., 1995). Thus, our data showed that formulation and concentration used may be accelerating the outbreak of the animals. In addition, glyphosate induces fragility in the structure of the chorion, which facilitates its rupture, inducing an increase in the hatching rate in zebrafish embryos (Zhang et al., 2017). This is a worrying factor since it is a process that occurs through biochemical and physical mechanisms (Wang et al., 2018). Also, hatching can be related to body movements, which are necessary to break the egg envelope and can be associated with loss of muscle capacity due to abnormal body formation (Costa-Silva et al., 2018). However, in our study, the number of spontaneous movements was not affected by exposure to glyphosate.

Developmental abnormalities are important parameters for the evaluation of pesticide toxicity. We found malformations in embryos for all tested concentrations of herbicides, which we categorized as pericardial edema, yolk sac edema, spinal curvature, and body malformations (including tail malformation, short tail, and head malformation). Edema in the yolk sac has been observed since the beginning of embryonic development, this is because the metabolic system is deficient during the first two days, indicating that glyphosate may be accumulating and causing edema (Wu et al., 2017). This ability of organophosphates (*e.g.* glyphosate) to cause edema in the yolk sac in zebrafish embryos has been proven by several studies (Pamanji et al., 2015; Sulukan et al., 2017; Suvarchala and Philip, 2016). We observed that at a concentration of 500 µg / L there was a greater number of animals with malformations. On the other hand, in

another research it was found the presence of only malformations in the body at a concentration commercial formulation of glyphosate not specificated of 1 mg/L (1000 µg/L), the heart, yolk sac, and spine are not affected (Sulukan et al., 2017). In our study, after 24 hpf it was already possible to observe edema in the yolk sac, which can block the supply of nutrients during embryonic development, affecting cardiac function and the development of other organs. The heart is the first functional organ to form, playing a pivotal role during the development of a zebrafish embryo (Fishman and Chien, 1997; Stainier et al., 1993). Furthermore, it can result in several cardiac abnormalities, such as low heart rate and malformation.

Regarding changes in heart rate caused by glyphosate, we can see that tachycardia occurred in groups exposed to the herbicide, as predicted due to cardiac abnormalities. Other studies have found a decrease in the heart rate of fish exposed to this herbicide (Gaur and Bhargava, 2019; Lanzarin et al., 2019). The decrease in heart rate may be associated with genes involved in the contraction and excitation of the heart, altering calcium homeostasis through the expression of the proteins involved and also the oxide-nitric pathway (Gaur and Bhargava, 2019). As for the locomotor behavior assessed by the touch response test, we found no damage caused by glyphosate. Locomotor behaviors play an important role in the development of zebrafish, such as incubation, escape behavior, feeding, social and defensive activities (Colwill and Creton, 2011). Bearing in mind that locomotion will directly affect the animals' survival, further tests are necessary to prove that this GBH in these concentrations does not affect the embryos.

In this study, no changes were observed in the parameters related to oxidative stress (activity of the enzyme catalase, the total antioxidant capacity against peroxyl radicals) in embryos exposed to glyphosate because there seems to have been no increase in the production of ROS (especially peroxyl radicals). However, there was a reduction in the activity of the GST enzyme, responsible for the detoxification of the body's xenobiotics, in the GLY 250 and GLY 500 groups. According (Diken et al., 2017) in an in vitro study, the herbicide glyphosate was able to inhibit GST activity in a non-competitive way, that is, glyphosate affected the catalytic function of GST but not it is binding to the substrate (such as reduced glutathione, GSH). Thus, impairment in catalytic functions reveals a large number of harmful agents, such as herbicides, insecticides, oxidative stress products, and many environmental toxic elements, which will have their actions facilitated.

Glyphosate is classified as an organophosphate, a class widely known for inhibiting enzymes, such as GST and AChE (Sandoval-Herrera et al., 2019). The activity of

acetylcholinesterase is affected by the binding of organophosphates, such as glyphosate, where this enzyme undergoes a conformational change at the acetylcholine binding site (Braitberg, 2019). In our research, the enzyme AChE had its activity reduced in the GLY 250 group. That ends up accumulating, resulting in over-stimulation of nicotinic and muscarinic ACh receptors and impeded neurotransmission (Čolović et al., 2013). This reduction in GST activity and AChE activity due to exposure to different glyphosate formulations has also been observed in vivo in tadpoles *Rhinella arenarum* (Lajmanovich et al., 2011) and neotropical fish *Prochilodus lineatus* (Modesto and Martinez, 2010), but higher concentrations were used than 250-1000 µg/L, that is, greater than the range of our experiment. We also found that a study evaluated the effect of glyphosate on these enzymes, but the concentrations used were higher than 1000 µg/L (Lanzarin et al., 2019). In this study, the concentration of 250 µg/L of glyphosate, half of the acceptable concentration for drinking water described in Brazilian legislation, was able to inhibit enzymes important for the normal metabolism of developing zebrafish embryos. Chronic long-term exposure of glyphosate to fish in natural environments could cause relevant ecological disturbances (Giaquinto et al., 2017; Li et al., 2017). This leads us to infer that concentrations permitted by Brazilian law are toxic to non-target organisms such as zebrafish. This demonstrated susceptibility is strong evidence of damage to the health of an organism still in formation, as in the embryonic phase for many phylogenetic levels.

Therefore, in the present study, we can see that the herbicide based on glyphosate (Roundup Original DI®) in concentrations present in drinking water causes toxicity in the zebrafish embryo. Causing bodily malformations observed in all concentrations, effects on increased hatching, increased heart rate, and changes in the activity of two important enzymes. These data will allow us to better understand the potential toxic mechanism of glyphosate in the embryonic developmental stage of zebrafish. Both behavioral and molecular analysis can be useful to better understand the effects of these concentrations, especially those legally acceptable.

5. Conclusion

We conclude that the GBH concentrations allowed in drinking water for human consumption interfere in the embryonic and larval development of zebrafish. The impairment in all the natural stages of development evaluated is aggravated by the mortality rate and systemic and morphological changes. In addition, pronounced enzymatic dysfunctions were detected and related to biotransformation and neuroregulation, pointing to the worsening of

environmental contamination by this agent. Thus, the large-scale use of glyphosate, coupled with the permissiveness to its presence in human daily food, causes irreparable damage to the aquatic environment as it affects non-target animals such as zebrafish.

Notes

The authors have no competing interests to declare.

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Contributions

Jaíne Ames: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. Eduardo Stringini Severo: Methodology, Writing - Review & Editing. Dennis Guilherme da Costa-Silva: Methodology, Writing - Review & Editing. Tamiris Rosso Storck: Investigation, Writing - Review & Editing. Aline Monique Blank do Amaral: Investigation, Writing - Review & Editing. Antônio Azambuja Miragem: Conceptualization, Writing - Review & Editing, Visualization. Denis Broock Rosemberg: Investigation, Visualization. Vania Lucia Loro: Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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7. Figures legend

Figure 1. Effect of glyphosate concentration on mortality and embryonic development of zebrafish. The number of animals exposed to different concentrations of glyphosate (GBH 250, GBH 500, and GBH 1000) after 6, 24, 48, 72, and 96 hpf (n = 2 replicate exposures, each replicate containing 120 embryos). (A) Dead animals. (B) Hatching of embryos. Data are expressed as mean values ± SEM and analyzed by the two-way ANOVA test followed by a Tukey post-hoc test. * p <0.05; *** p <0.001; **** p <0.0001.

Figure 2. Malformations in the development of embryos of zebrafish exposed to glyphosate (GBH) at 72 hpf. All data are expressed as mean values ± SEM of 200 embryos analyzed individually for each group and analyzed by the one-way ANOVA test followed by a Tukey post-hoc test. * p <0.05; *** p <0.001; **** p <0.0001.

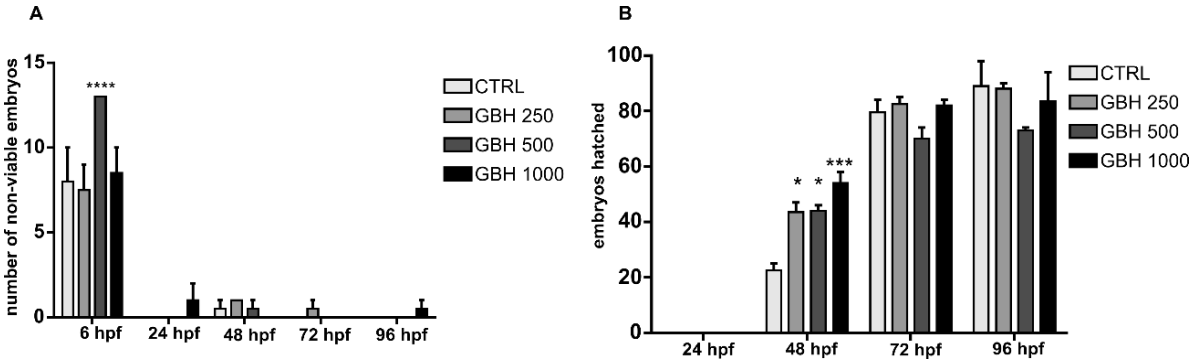
Figure 3. Malformations in the development of zebrafish embryos exposed to 250, 500, and 1000 µg/L of glyphosate at 24, 48, 72 and 96 hpf. Pericardial Edema (PE), Yolk Sac Edema (YSE), Body Malformations (BM) (including tail malformation, short tail, and head malformation), and Spinal Curvature (SC).

Figure 4. Effects of glyphosate on behavior. (A) Stimulus to touch expressed as the number of stimuli needed to swim first at 48 hpf. (B) Spontaneous movement of embryos at 28 hpf, expressed by curves/min These tests were performed in duplicate (n = 30 per group, in touch response; n = 15 per group, in spontaneous movement). Results are expressed as mean values ± SEM, statistically analyzed by the Kruskal-Wallis test and Dunn's Multiple Comparison Test. About the control, there was no statistical difference (p> 0.05).

Figure 5. Heart rate in the development of zebrafish embryos exposed to glyphosate (GLY) at 48 hpf. All data are expressed as mean values ± SEM of 15 embryos analyzed individually for each group in duplicate and analyzed by the one-way ANOVA test followed by a Tukey post-hoc test. **** p <0.0001.

Figure 6: Evaluation of biochemical parameters in zebrafish embryos with 96 hpf at different glyphosate concentrations. A) Protein concentration; B) Thiobarbituric Acid Reactive Substances Test (TBARS); C) Determination of Reactive Oxygen Species (ROS); D) Antioxidant Capacity Against Peroxides (ACAP); E) Glutathione S-Transferase; F) Acetylcholinesterase; G) Catalase; H) NPSH levels. Data were analyzed using one-way ANOVA followed by a Tukey post-hoc test. * p <0.05; ** p <0.01.

8. Figures



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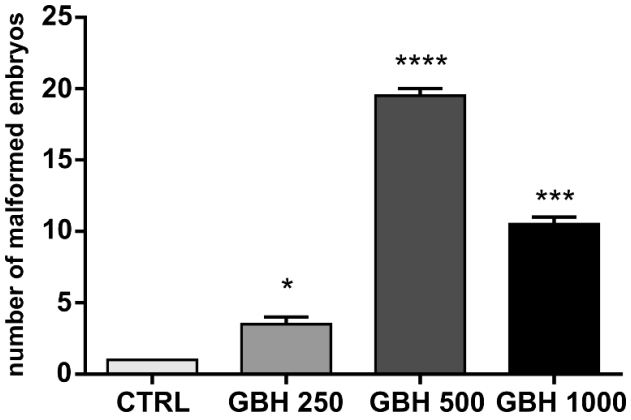

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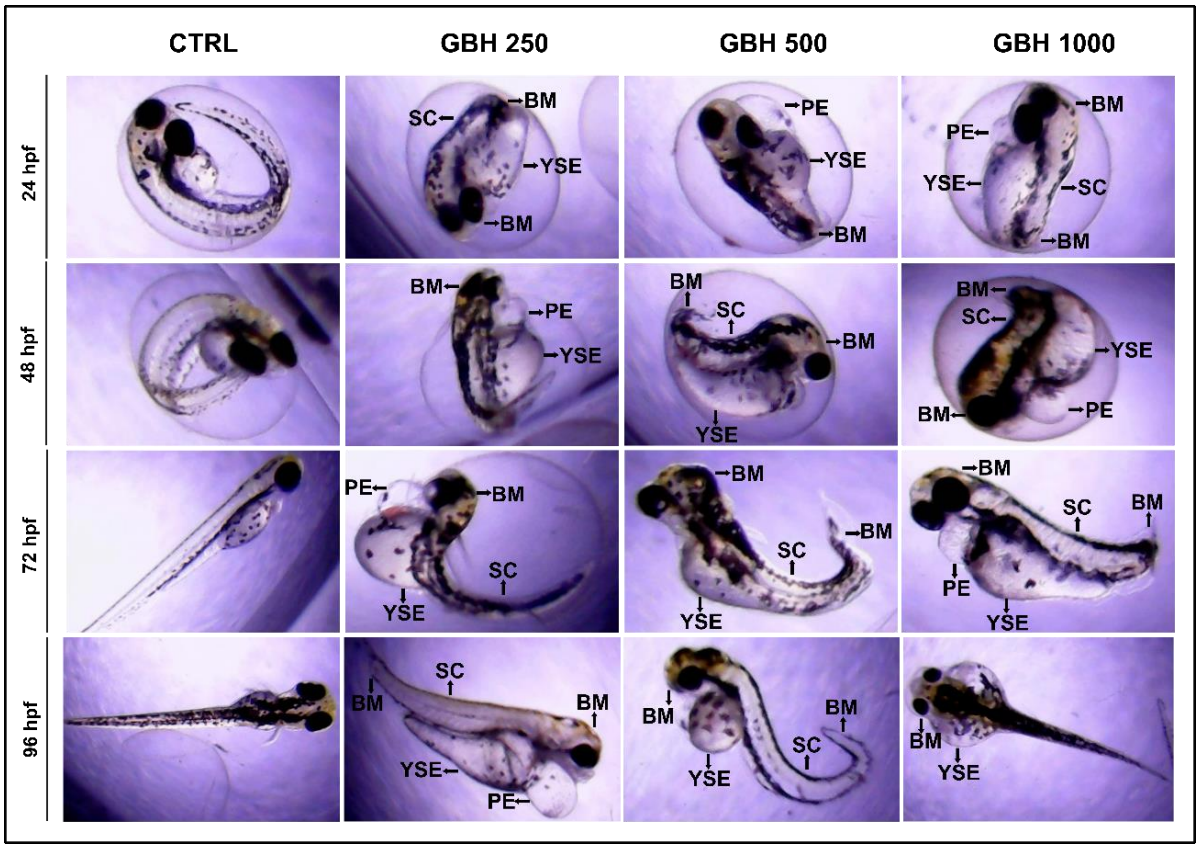

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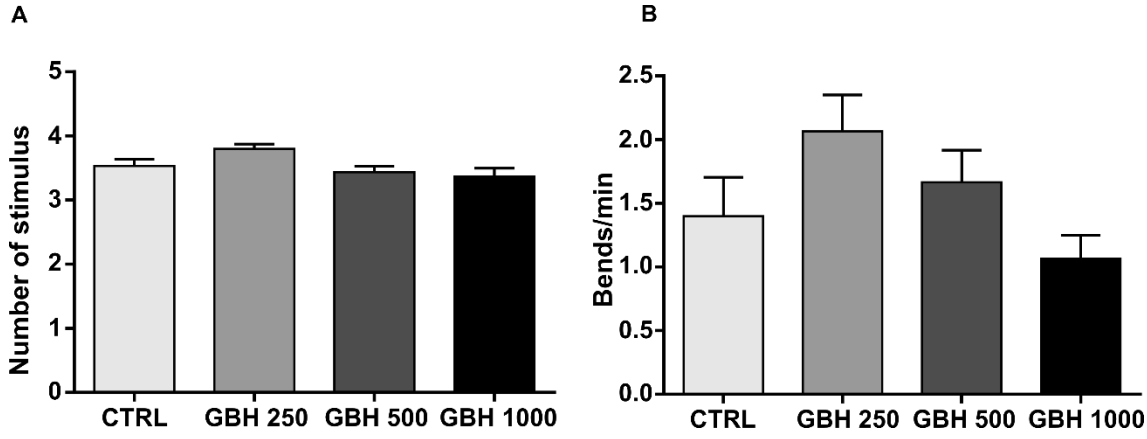
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1
2
3
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5
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GBH 500
GBH 1000
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Fig 4.

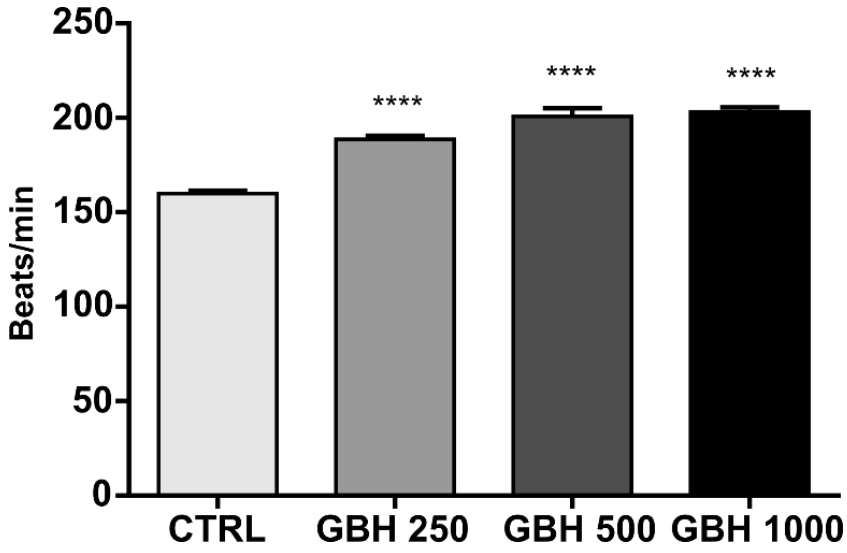
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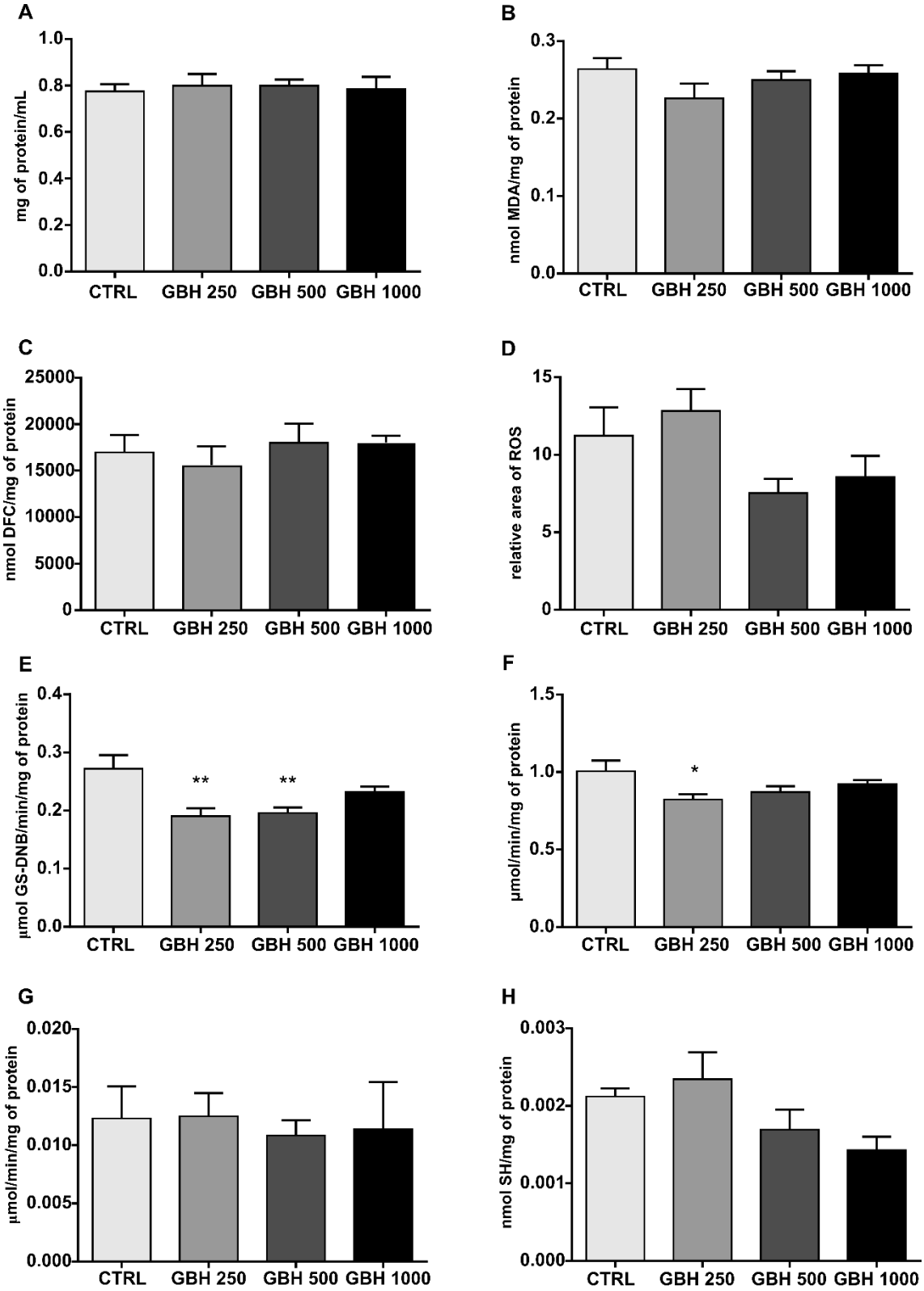
Fig 5.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mg of protein/mL
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0.3
0.2
0.1
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CTRL
GBH 250
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C
nmol DFC/mg of protein
25000
20000
15000
10000
5000
0
CTRL
GBH 250
GBH 500
GBH 1000
D
relative area of ROS
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5
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E
µmol GS-DNB/min/mg of protein
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F
µmol/min/mg of protein
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G
µmol/min/mg of protein
0.020
0.015
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0.005
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CTRL
GBH 250
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GBH 1000
H
nmol SH/mg of protein
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0.001
0.000
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GBH 250
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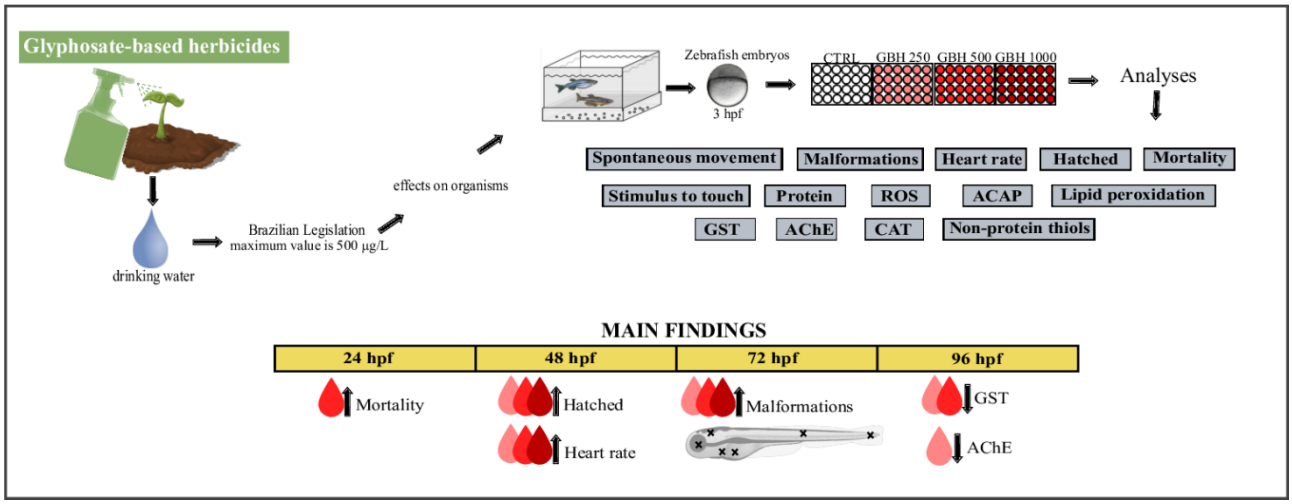
Fig 6:

GRAPHICAL ABSTRACT



5 CAPÍTULO 2: EFEITOS DO GLIFOSATO NO PEIXE-ZEBRA (*Danio rerio*): UMA REVISÃO SISTEMÁTICA E META-ANÁLISE

Este capítulo revisa os artigos de pesquisa existentes que têm como foco os danos causados pelo glifosato nas fases embrionária, larval e adulta do peixe-zebra. Após uma busca intensa, obtivemos um total de 20 artigos em dois bancos de dados. Após a seleção dos artigos realizamos uma meta-análise e uma meta-regressão. Este estudo gerou o seguinte artigo a ser submetido para a publicação na revista "*Environmental Pollution*".

EFFECTS OF GLYPHOSATE ON ZEBRAFISH (*Danio rerio*): A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT: Glyphosate herbicide is widely used in agriculture around the world. Thus, its active ingredient and adjuvants end up reaching the aquatic ecosystem and putting fish species at risk. Therefore, this study aimed to evaluate the effects of glyphosate on the embryonic, larval and adult stages of zebrafish (*Danio rerio*) through a meta-analysis. For this, a search was performed in the PubMed and Science Direct databases. Articles evaluating the effect of glyphosate on zebrafish published up to July 2020 were considered. After obtaining the data, the R software was used to perform the meta-analytical tests. Selection by title, abstract and full text was performed, and 20 original research articles with zebrafish exposed to commercial and/or pure glyphosate were obtained. We observed that embryos exposed to glyphosate exhibit an increase in mortality by 3 hpf (I^2 = 63%; $p < 0.01$), 24 hpf (I^2 = 91%; $p < 0.01$), 48 hpf (I^2 = 93%; $p<0.01$, 72 hpf (I^2 = 95%; $p<0.01$) and 96 hpf (I^2 = 75%; $p<0.01$). In hatching rates there was an increase in 48 hpf (I^2 = 72%; $p < 0.01$), a decrease in 72 hpf (I^2 = 96%; $p < 0.01$) and there was no difference in 96 hpf (I^2 = 0 %; $p = 0.99$). The malformations revealed an increase in the number of morphological abnormalities related to yolk sac edema (I^2 = 80%; $p < 0.01$), pericardial edema (I^2 = 75%; $p < 0.01$), spinal curvature (I^2 = 77 %; $p<0.01$) and body malformations (I^2 = 88%; $p<0.01$). Regarding morphology, we observed a decrease in body size (I^2 = 99%; $p < 0.01$). Regarding heart rate, there was a decrease in the number of beats by 48 hpf (I^2 = 100%; $p <0.01$) and 72 hpf (I^2 = 100%; $p = 0$). The biochemical results demonstrated an increase in ROS in the gills within 24 hours of exposure to glyphosate (I^2 = 88%; $p < 0.01$) and increased ACAP in the gills within 96 hours of exposure (I^2 = 61%; p = 0, 05). We verified that glyphosate decreased the distance covered (I^2 = 99%; $p <0.01$) and the mean speed of the animals (I^2 = 88%; $p <0.01$) and increased the number of rotations (I^2 = 98%; $p < 0.01$). We conclude with this work that our results indicate damage caused in the embryonic, larval and adult stages of this fish species.

Keywords: embryo, larval, adult, herbicide, ecotoxicology.

1. Introduction

The demand and production of pesticides to control unwanted organisms has increased annually worldwide (Zhang and Liu, 2017), mainly motivated by the advance of agricultural technology and greater productivity. Although pesticides may benefit agricultural production, there is a plethora of ecosystem damage routinely recorded in studies (Lanzarin et al., 2020; Rumschlag et al., 2020; Ruuskanen et al., 2020; Sulukan et al., 2017; Zhang et al., 2017).

One of the most used pesticides to remove unwanted plants (i.e. herbicide) is glyphosate (Glyphosate-based herbicides - GBH). Since its first commercialization in the 70s (Baird, 1971), GBH has been the most widely used herbicide in the world (Benbrook, 2016). For example, until 2014, around 800 million kg of GBH were used worldwide, with estimates expected to reach 740 to 920 thousand tons by 2025 (Benbrook, 2016; Maggi et al., 2020). In agriculture, the glyphosate is mixed with chemical ingredients, such as adjuvants and surfactants (e.g. polyoxyethylene amine - POEA and alkyl polyphosphate amine) (FAO, 2016). When disposed in the natural environment, the glyphosate can be decomposed into two

metabolite types: aminomethylphosphonic acid (AMPA) or sarcosine (Borggaard and Gimsing, 2008).

The intensified use of glyphosate in agricultural activities, including surfactants, adjuvants, and its main metabolite, contributes to the increase of these compounds in the ecosystem (Benbrook, 2016). This herbicide can be found in the aquatic environment through releasing, inadequate packaging disposal, wind, spray diversions, runoff (Silva and Fay, 2004). In fact, glyphosate and AMPA have been frequently found in the seawater, urban streams, wetlands, surface water, groundwater, freshwater, and water bodies (Aparicio et al., 2013; Coupe et al., 2012; Mercurio et al., 2014; Okada et al., 2018; Ruiz-Toledo et al., 2014). According to the literature, concentrations of 0.70 mg L^{-1} can be observed on the water surface (Annett et al., 2014; Byer et al., 2008; Peruzzo et al., 2008). When glyphosate comes into contact with the aquatic environment, it can affect the quality of water, aquatic plants and animals (Dornelles and Oliveira, 2014; Hong et al., 2018; Liu et al., 2019; Moreno et al., 2014; Vera et al., 2010).

More specifically in fishes, the glyphosate can be absorbed by gills and via a dietary route, during all stages of life (Hued et al., 2012). Glyphosate toxicity can cause oxidative stress, affect the activity of antioxidant enzymes and inhibit activity is acetylcholinesterase (AChE) (Glusczak et al., 2011, 2007; Lushchak et al., 2009). In this sense, the use of zebrafish (*Danio rerio* Hamilton 1822) represents an adequate alternative to study the effects of pesticides on water. Zebrafish is an ideal bioindicator of water quality, which exhibits a rapid development, size suitable for maintenance (3 to 4 centimeters), high fertility, and meets the 3 Rs criterion (Grunwald and Eisen, 2002). Another feature intrinsically associated with zebrafish is that different stages of development may be evaluated in toxicological studies (embryo: 0-48 hours; larva: 2-30 days; adult: 2-3 months). In the embryonic and larval phases, some of the advantages are easy handling and observation of structures (transparent animals), along with rapid development (OECD, 2013). Therefore, several characteristics of this animal are highly favorable for use in ecotoxicological studies.

Although specific studies have revealed effects of glyphosate on zebrafish (Fiorino, 2018; Lanzarin et al., 2019; Moraes et al., 2020; Sulukan et al., 2017), a broad and systematic view remains largely neglected. Therefore, a framework that integrates sophisticated analytical approaches to systematically assessing the effects of glyphosate can provide an overview of zebrafish sensitivity to the herbicide, with implications relevant to freshwater ecosystems as a whole. Here we performed a meta-analysis of glyphosate toxicity on both the embryonic, larval

and adult stages of zebrafish to identify the effect of glyphosate in a broader spectrum. Specifically, we aimed to investigate 1) the extent to which glyphosate effects differ between stages; and 2) which routes are primarily involved in glyphosate toxicity. We provide a systematic view of glyphosate impacts on zebrafish models and our main findings clearly suggest that the extent of its toxicity may be stage-dependent.

2. Methods

2.1 Research sources, identification, and criteria for inclusion of studies

This meta-analytic review was conducted following the PRISMA guidelines for systematic reviews (Liberati et al., 2009). We carried out a PubMed and Science Direct full text search, which tested the effects of glyphosate on the embryo larval and adult stages of zebrafish.

We used the following search terms: “Glyphosate” AND “zebrafish” AND (“egg” OR “embryo” OR “larvae” OR “adult”), “Glyphosate” AND “danio rerio” AND (“egg” OR “embryo” OR “larvae” OR “adult”), “Roundup” AND “zebrafish” AND (“egg” OR “embryo” OR “larvae” OR “adult”) and “Roundup” AND “danio rerio” AND (“egg” OR “embryo” OR “larvae” OR “adult”). The search was carried out with no limited start date until July 2020, and the keywords were searched in English language. In addition, references from relevant publications (i.e. peer-reviewed articles) such as review articles, were checked to identify additional articles with data corresponding to the objectives of this study. We initially screened studies based on general titles and abstracts. Then, we read the full text to extract all the necessary information. This yielded a total of 1785 results that were read and searched for references associated with glyphosate and zebrafish (see Results section for more details).

Some studies were discarded because they had insufficient data for meta-analysis (e.g.), did not provide adequate information about the type of pesticide used, and/or studies had restricted access to the full text. No review, PhD theses, scientific note, or book chapter were included. We also disregarded articles in which the active ingredients of different pesticides were combined. In this sense, articles included met the following criteria: (1) original research, performed with zebrafish exposed to glyphosate; (2) glyphosate-based herbicides reported as one of the sources of exposure; (3) in vivo studies on zebrafish; and (4) results presented in mean and standard deviation (MD ± SD), mean and standard error (MD ± SE) and/or barplot type graphs.

Therefore, we only considered articles that included the previously mentioned criteria and directly addressed the effects of glyphosate on embryo-larval and adult stages. For the sake

of simplicity and statistical criteria (see Data analysis section), we group the data obtained into glyphosate effects caused on 1) mortality (e.g. in hours post fertilization), 2) hatched (e.g. in hours post fertilization), 3) malformation (e.g. yolk sac edema, pericardial edema, spinal curvature, body malformations), 4) morphology (e.g. body length), 5) heart rate (e.g. in hours post fertilization), 6) biochemistry (e.g. ACAP, ROS) of the animals, and 7) behavior (e.g. rotations, distance traveled, mean speed).

2.2 Data extraction

All search results considered were tabulated in a digital spreadsheet (Excel). This table was constructed containing the following information: authors, year of publication, country, stage of life, glyphosate formulation, concentration, replacement, dilution, the effect caused, specification of effect, and temperature. All extracted information was standardized to the same unit of measure (e.g. heart rate per minute, behavior expressed in m/s, etc.). Still, studies in which tested different concentrations of glyphosate for the same control (i.e. more than one intervention group) were considered as independent sampling units. Although different glyphosate formulations are known, we consider the combined effect of different commercial and pure formulations because the data are insufficient to perform separate meta-analyses. Yet, both the active ingredient glyphosate and its adjuvants can be found together in the aquatic environment (ref). Therefore, we treat throughout the manuscript the results considering the combined formulations, as similarly conducted in previous studies (see Battisti et al., 2021 for similar approaches).

For each article, we also extracted the sample size (n), mean, and standard deviation for both control and experimental group. When the results were presented in standard error of the mean (SEM, standard error of the mean), we converted this into standard deviation (SD) using the equation 1 (Vesterinen et al., 2014). This procedure was performed using the FIJI software (ImageJ), whereby it consists in extracting data from a graph with the presence of error bars and without their overlapping (Schindelin et al., 2012). The method adopted followed the same principle as that used by specialized software such as UnGraph, Data Thief and GraphClick, all considered reliable (Flower et al., 2016; Shadish et al., 2009). This method consists of loading an image of the graph into the program, performing the program scale calibration from the ordinate axis scale and extracting, from the drawn straight lines, the mean and standard deviation or error values.

$$SD = SEM \times \sqrt{n}$$

Equation 1. Calculation to acquire the standard deviation of the data. Wherever, *SD*=standard deviation, *SEM*=standard error of the mean and *n*=sample size.

2.3 Data analysis

We performed a meta-analysis on the sample size, mean, and standard deviation in order to calculate a standardized measure for each study (i.e. effect size). We then used the R package meta (Balduzzi et al., 2019; Schwarzer, 2007) to calculate the standardized mean difference (SMD) as an estimate of effect size on two types of outcomes: continuous and binary data. We consider continuous outcomes those in which studies have evaluated the effect of glyphosate on quantitatively measured structures. For example, heart rate was measured across studies by the number of heart beats per minute. In contrast, we consider binary outcomes to be those that have been categorically measured. For example, the number of animals that died was counted in mortality.

For continuous outcomes (adult: biochemistry and behavior; embryo and larva: heart rate and morphology), the SMD was estimated using Hedges' g statistic (Hedges and Olkin, 1985) and the between-study variance (τ^2) was calculated using the DerSimonian-Laird (DL) method (Dersimonian and Laird, 1986). Hedges' g expresses the difference of the means in units of the pooled standard deviation and is highly recommended in meta-analysis because it has a lower Type I error. The SMD (effect size) was considered significant when the 95% confidence interval (CI) did not include zero. We also quantify and test for statistical heterogeneity using Higgin's I^2 (Higgins et al., 2003), which the heterogeneity values range from 0 to 100% (<25% = low, 25-75% = moderate, >75% = high). Still, we considered a sub-group analyses to determine the effects of glyphosate based on the hours post fertilization (hpf) or some specification parameter dependent (see forest plot legends for more details on the sub-group used). Calculated effect sizes and 95% CI were used to generate forest plots. Results were considered significant at the level of the $p<0.05$. All these procedures involving continuous outcome data were implemented in the "metacont" R function.

For binary outcomes data (embryo and larva: mortality, hatching rate and malformation), we calculated the fixed and random effects estimates for meta-analysis using the "metabin" R function. Specifically, we used the Mantel Haenszel (MH) method with the Hedges estimator of between-study variance (τ^2) to calculate Odds ratio (OR) with a 95% CI (Mantel and Haenszel, 1959; Egger et al., 2001). Results were considered significant at the level of the $p<0.05$. We also evaluate the degree of residual heterogeneity in our data using Higgin's

I^2 statistics (Higgins et al., 2003). Similarly to continuous outcomes data, a sub-group analysis was performed.

To examine the magnitude and direction of the effects of glyphosate concentration level on embryonic-larval and adult stages, we performed meta-regression models using the "metareg" function. We checked the publication bias in our dataset from three approaches. First, we analyzed ten items checklist of the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE), which are mainly associated with the experimental procedures used in each study (Hooijmans et al., 2014). Second, we graphically inspected asymmetry using funnel plots (R funnel function). Third, we performed the Egger regression test (R "metabias" function), which consists in a quantitative method to test for asymmetry in the funnel plot (Egger et al., 1997). All the above functions were from the "meta" package (Balduzzi et al., 2019) in R version 4.0.3 (R Core Team 2021).

3. Results

3.1 Systematic search

Database searches resulted in an initial total of 1785 documents (n=149 from PubMed, and n=1636 from Science Direct) were obtained through systematic search (Figure 1). Duplicates were removed (n=847) and other research subtypes such as reviews and book chapters (n=764). Furthermore, studies that analyzed the effects on other living beings were rejected (n=165) and studies with other contaminants without glyphosate (n=23). After these exclusions, we obtained a total of 31 articles, which were subjected to full text reading. In addition, in vitro studies (n = 8) evaluated the effects of exposure of zebrafish cells to glyphosate, effects of other variables with glyphosate, such as pH and sediment (n = 2), and without statistical analysis (n = 1). After selection by title, abstract and full text, 20 original research articles with zebrafish exposed to glyphosate commercial and/or pure were obtained. All these studies were included in the systematic review, the main characteristics and results are presented in Table 01.

3.2 Study characteristics

Taken together, the studies included in this review were published between 2014 and 2020, carried out in Brazil (n=11), China (n=1), India (n=1), Portugal (n=2), Turkey (n=1), United Kingdom (n=1) =1), Czech Republic (n=1) and United States (n=2). The articles are detailed in Table 01. Of these, 12 articles used zebrafish in the embryonic and larval stages, and

8 studies in the adult stage, one of which evaluated the embryonic, larval and adult stages. Dosages of pure or commercial glyphosate used ranged from 0.01 to 600 mg/L. The pure formulations used ranged around 99% pure and the commercial formulations were quite diverse. All selected studies show that exposure of zebrafish, regardless of its stage of life, to some GBHs or pure glyphosate induces damage to this animal.

The findings showed that in the embryonic and larval stages, glyphosate affects development. Regarding the mortality and hatching rate of embryos and larvae, the studies evaluated these effects through exposure to commercial and pure glyphosate at concentrations ranging from 0.01 to 100 mg/L (de Brito Rodrigues et al., 2019, 2017; Fiorino, 2018; Lanzarin et al., 2020, 2019; Sulukan et al., 2017; Uren Webster et al., 2014). The heart rate was also verified to check cardiovascular damage (Gaur and Bhargava, 2019; Lanzarin et al., 2019; Roy et al., 2016b). Changes in morphology and malformations were evaluated through exposure to commercial and pure glyphosate ranging from 0.1 to 600 mg/L (de Brito Rodrigues et al., 2019; Roy et al., 2016a, 2016b; Sulukan et al., 2017; Zhang et al., 2017). Biochemical damage was found in three articles, through the analysis of antioxidant enzymes, proteins and other biomarkers (Lanzarin et al., 2019; Panetto et al., 2019; Sulukan et al., 2017; Zhang et al., 2017). In addition, they also verified the effects on behavior, through changes in locomotor activity, aversive behavior, rotation and other analyses (Bridi et al., 2017; Zhang et al., 2017).

Regarding the effects of glyphosate on adult zebrafish, biochemical and behavioral changes were verified. In the biochemical analysis, the authors found damages in the gills, liver, brain and muscle in biomarkers such as SOD, CAT, GPx, ROS, ACAP, AChE and changes in the expression of some genes (Jaramillo et al., 2018; Lopes et al., 2018, 2014; Moraes et al., 2020; Santo et al., 2018; Velasques et al., 2016). Also, the authors noted effects on behavior, such as distance traveled, latency to enter the upper zone, average speed, rotations and distance covered (Bridi et al., 2017; da Costa Chaulet et al., 2019; da Rosa et al., 2016).

3.3 Study quality and risk of bias

Figures 2A and 2B present as percentages of “high” or “low” risk of bias for each domain assessed for risk of bias within each individual study and across studies, respectively. In 100% of the studies (n=20), there was an adequate distribution of the animals between the groups and they were similar at the beginning of the treatment. There is a low risk of bias in these two factors, demonstrating a homogeneity of animals between groups. As for allocation secrecy, 100% of the studies (n=20) did not report whether the group allocation was adequately

concealed, and 65% of the studies (n=13) did not mention the random housing of the animals. The random allocation of animals is not yet a standard practice in animal experiments, and may reflect a possible distortion of the global interpretation of the data. In all studies (100%, n=20) there were no reports about the lack of knowledge about the interventions by caregivers, nor about the results being collected randomly and by a blind observer. Usually, in experimental designs with animal models, these procedures are rarely described. All studies (100%, n=20) did not present other potential sources of bias and also incomplete results. In this sense, the objects of comparison (variables) were potentially approximated and analyzed with greater certainty. Finally, 80% of the studies (n=17) had a conclusion free of selectivity. Thus, we started with the statistical procedures described, in order to build a body of evidence on the problem in question.

3.4 Meta-analysis

3.4.1 Glyphosate affects mortality and hatching in zebrafish embryos

We observed that embryos exposed to glyphosate exhibit an increase in mortality in 3 hpf (I^2 = 63%; p<0.01), 24 hpf (I^2 = 91%; p<0.01), 48 hpf (I^2 = 93%; p<0.01), 72 hpf (I^2 = 95%; p<0.01), and 96 hpf (I^2 = 75%; p<0.01) (Figure 3). Thus, the number of dead animals during the entire embryonic and larval stage is higher in groups exposed to this pesticide. Visual inspection of the funnel plot in Supplementary Figure 1A and the Egger test (t=2.139, p=0.0374) indicate a possible presence of publication bias in our results. To check whether there was a relationship between mortality and the dosage of glyphosate or GBH used, we performed a meta-regression. The target regression demonstrated a significant positive relationship between embryo mortality and the dosage used (se=0.0031, p<0.0001, Supplementary Figure 2A). Showing that the higher the concentration of glyphosate used, there is a greater probability of observing an effect on mortality.

We found mixed results regarding the effects of glyphosate on hatching rates, whereby increased in 48 hpf (I^2 = 72%; p<0.01), decreased in 72 hpf (I^2 = 96%; p<0.01) and there was no difference in 96 hpf (I^2 = 0%; p=0.99) (Figure 4). We observed that at 48 hpf glyphosate accelerates the hatching of embryos. Visual inspection of the funnel plot (Supplementary Figure 1B) and the Egger test (t=1.843, p=0.0736) indicate no evidence of publication bias in our results. In the meta-regression we observed that it showed a significant positive relationship

between the hatch rate and the dosage of glyphosate or GBH used (se=0.0029, p=0.0035, Supplementary Figure 2B).

3.4.2 Glyphosate causes malformations and structural abnormalities in zebrafish embryos.

Our results on malformations revealed an increase in the number of morphological abnormalities related to the yolk sac edema (I^2 = 80%; p<0.01), pericardial edema (I^2 = 75%; p<0.01), spinal curvature (I^2 = 77%; p<0.01) and body malformations (I^2 = 88%; p<0.01) (Figure 5). Visual inspection of the funnel plot (Supplementary Figure 1C) and the Egger test (t=1.34; p=0.1919) indicate no evidence of publication bias in our results. Meta-regression demonstrated a significant positive relationship in glyphosate-exposed embryos in malformation (se=0.0066, p=0.0002, Supplementary Figure 2C).

In relation to morphology, we regarded changes mainly associated with a decrease in body size in animals exposed to glyphosate (I2 = 99%; p<0.01, Figure 6). Visual inspection of the funnel plot (Supplementary Figure 1D) and the Egger test (t=-2.359, p<0.0001) indicate the possible presence of publication bias in our results. Meta-regression demonstrated a significant positive relationship in glyphosate-exposed embryos on morphology (se=0.3835, p<0.0001, Supplementary Figure 2D).

3.4.3 Glyphosate alters heart rate in zebrafish embryos.

Our findings on the heart rate of embryos exposed to glyphosate indicate a decrease in the number of beats in both 48 hpf (I^2 = 100%; p<0.01) and 72 hpf (I^2 = 100%; p=0) (Figure 7). Visual inspection of the funnel plot (Supplementary Figure 1E) and the Egger test (t=4.751, p=0.0014) indicate the possible presence of publication bias in our results. Meta-regression demonstrated a significant negative relationship in glyphosate-exposed embryos on heart rate (se=0.0493, p<0.0001, Supplementary Figure 2E). We found that, at lower concentrations, there is a greater likelihood that we will see an effect on heart rate.

3.4.4 Glyphosate changes the reactive oxygen species and antioxidant capacity against peroxyl radicals in the gills of zebrafish adults.

The biochemical results demonstrated an increase in reactive oxygen species (ROS) in the gills within 24 hours of exposure to glyphosate (I^2 = 88%; p<0.01; Figure 8). Also, it increased the antioxidant capacity against peroxyl radicals (ACAP) in the gills in 96 hours of

exposure (I^2 = 61%; p=0.05). Visual inspection of the funnel plot (Supplementary Figure 1F) and the Egger test (t=-2.359, p=0.0333) indicate the possible presence of publication bias in our results. The meta-regression did not demonstrate a significant response in relation to the biochemical analyzes and concentration used (se=0.2283, p=0.8835, Supplementary Figure 2F).

3.4.5 Glyphosate alters the behavior of adult zebrafish.

We found that glyphosate decreased the distance traveled (I^2 = 99%; p<0.01) and the average speed of the animals (I^2 = 88%; p<0.01). In addition, increased the number of rotations (I^2 = 98%; p<0.01) (Figure 9). Visual inspection of the funnel plot (Supplementary Figure 1G) and the Egger test (t= 2.368, p=0.0280) indicate the possible presence of publication bias in our results. The meta-regression demonstrated a significant positive relationship between adult behavior and the concentration used (se=0.6598, p<0.0001, Supplementary Figure 2G). Showing that the higher the concentration of glyphosate used, there is a greater probability of observing an effect on behavior.

4. Discussion

The damage caused by the toxic effects of glyphosate and its additives are widely reported in the *corpus* of scientific literature. The use of zebrafish (*Danio rerio*) as a model for mechanistic description of its action, as well as the ecological implications caused by this pesticide, have increased considerably in recent years. Thus, we chose to use the results of works published until 2020 with this species *in vivo*, compiling the stages of its life cycle (embryonic, larval and adult). Studies showing that glyphosate-based herbicides affect the life cycle of species *Cantareus aspersus*, *Lithobates sylvaticus* and *Chrysoperla externa* (Druart et al., 2017; Lanctôt et al., 2014; Schneider et al., 2009), which demonstrates the need for a broader look at these losses. However, there was no record of experimental trials that considered the damage caused by glyphosate at all stages of zebrafish life. In the natural environment, the fish will come into contact with the pesticide at all stages of its life.

Due to the chronological variation of glyphosate applications in crops and its permanence of about 60 days in surface waters, studies have shown its prevalent presence in aquatic ecosystems (Annett et al., 2014; Pohl et al., 2019). In the US, maximum concentrations of 73 μg/L were found in rivers and streams, <0.02 in lakes, and 301 μg/L in swamps (Battaglin et al., 2014). Measured concentrations of glyphosate in surface freshwater ranged from 2.7 to

10.3 mg acid equivalent/L (Córdova López et al., 2019; Ronco et al., 2016). These data raise important concerns about the constant presence and high solubility of glyphosate, as well as its potential threat caused by exposure to non-target organisms present in the environment.

Exposure of fish embryos to GBH presents increased danger, since the chorionic membrane of the embryo is the first to come into contact with the toxic agent. This chorion is an acellular envelope, which is known to be about 0.5–0.7μm thick with three layers perforated by pore channels in fertilized eggs (Bonsignorio et al., 1996; Rawson et al., 2000). It acts as a barrier to protect embryos from external stimuli. (Tran et al., 2021). In addition, the chorion allows molecules to pass into the embryo by passive diffusion (Berghmans et al., 2008). However, the membrane has no protective effect on the development of embryos exposed to organophosphates, which penetrate the chorion and cause lethal effects (Ansari and Ahmad, 2010). Objectively, glyphosate induces changes in the chorion structure (Zhang et al., 2017). This occurs because glyphosate accesses the embryo through the chorion pores, mainly due to its low molecular weight, high polarity, low solubility in organic solvents and high solubility in water (Sanchís et al., 2012). This entry into the chorion causes glyphosate and/or additives present in GBHs to be absorbed by the developing embryo.

In the early period of life, the embryo is in the cleavage and blastula stage, and this is when cell division occurs (Kimmel et al., 1995). Because of this, this period becomes the most critical for the survival of the embryo itself. The high sensitivity of the organism in this period, the high toxicity to glyphosate was evident. We observed in our meta-analysis that up to 3 hpf there is an increase in mortality in animals exposed to 0.01 to 15 mg/L of glyphosate. In another study, there was an increase in mortality in embryos (3 hpf) exposed to 15 mg/L of commercial glyphosate (Lanzarin et al., 2019). It is important to highlight that in the natural environment there are more environmental interferences, in addition to glyphosate, however, this increase in challenges to embryonic survival can lead to population suppression.

During gastrulation (24 hpf), we found an increase in mortality in animals exposed to 0.01 - 400 mg/L of glyphosate. There was a pronounced increase in mortality in embryos with 22 hpf, exposed to 8.5 and 15 mg/L of commercial glyphosate (Lanzarin et al., 2019). This high mortality is related to developmental delay and embryo malformations. It was evident in our work that embryos exposed to glyphosate have a smaller body size. This evidence is reinforced in a study that evaluated gene expression *ntl (no tail)*, responsible for the formation of the notochord, which demonstrated that glyphosate can reduce the structure of the notochord related to the smaller size of the body (Odenthal et al., 1996; Zhang et al., 2017; Zou et al.,

2009). We observed a dose-effect relationship, such that the higher the concentration of glyphosate used, the greater the likelihood of anatomical changes, such as body length.

Regarding malformations, the results of our meta-analysis demonstrate an increase in the number of animals with embryonic alterations when exposed to glyphosate. Furthermore, our evidence suggests that the higher the concentration of glyphosate used, the greater the probability of observing these anatomical damages. Interestingly, these malformations, induced by glyphosate, are edema in the yolk and pericardial sac, spinal curvature and malformations in the body (head, eye and tail). We demonstrate that, based on the experimental studies analyzed, it is very frequent to record morphological abnormalities in a wide spectrum of pure glyphosate concentrations, including 1-100 mg/L (Sulukan et al., 2017), 100 mg/L (Gaur and Bhargava, 2019) and 8.5 mg/L (Lanzarin et al., 2019).

Probably, the reason for the formation of edema in the yolk and pericardial sac in exposed embryos could be a deficiency of the metabolic system associated with the accumulation of the pesticide (Wu et al., 2017). Or, these edemas can occur due to the inhibition of genes *slc2a10/glut10* (Solute carrier family 2 member 10/Glucose transporter 10) or *Lrrc10* (Leucine-rich Repeat Containing protein 10) (Kim et al., 2007; Willaert et al., 2012). We can relate edema to interference in the synthesis and metabolism of lipids present in the yolk sac. Thus, due to this difficulty in absorbing lipids, which are important for the survival of animals, there was a basic dietary deficiency for the development of organs and, consequently, malformations in the body. Regarding the damage to the formation of the spine, we can list: i) an over-expression of growth hormone (Carlos et al., 2010); ii) decreased collagen in the spine (Çelik et al., 2012); iii) inhibition of gene expression *col27a1a* (collagen, type XXVII, alpha 1a) and *col27a1b* (collagen, type XXVII, alpha 1b) (Christiansen et al., 2009); iv) lysyl oxidase inhibition (Snawder and Chambers, 1993); e v) suppression or *down regulation* of the gene *ptk7* (protein-tyrosine kinase-7) (Hayes et al., 2014).

The effects of morphological changes can affect locomotion. May cause changes in locomotor activity and aversive behavior after exposure to pure glyphosate or Roundup® commercial formulation, at concentrations of 0.01, 0.065 and 0.5 mg/L (Bridi et al., 2017). By affecting the animal's morphology in the embryonic and larval stages, it can cause several damages, such as locomotor difficulties. Thus, it increases the susceptibility to predation, difficulty in searching for food, and in finding partners for reproduction. These factors promote impaired development, which can lead to death.

In our review, we found that glyphosate increases mortality, accelerates hatching, and decreases the heart rate of glyphosate-exposed embryos by 48 hpf. Regarding mortality, this was increased in embryos exposed to concentrations between 2 and 400 mg/L. Another study also demonstrated an increase in mortality in 48 hpf embryos exposed to similar concentrations of 8.5 mg/L and 15 mg/L of commercial glyphosate (Lanzarin et al., 2019).

Regarding the hatching rate, we present strong evidence that there is a pronounced increase in the number of hatched animals at 48 hpf, when exposed to concentrations between 0.01mg/L and 400 mg/L. Our findings allowed us to observe that the higher the concentration of glyphosate used, the greater the likelihood of hatch implications. An interesting study showed that at 48 hpf there was an increase in the number of animals hatched in two formulations of commercial glyphosate, at concentrations of 1.8, 3.6, 8.3 and 18 mg/L (de Brito Rodrigues et al., 2017). This hatch corresponds to the release of individuals from the chorion envelope and marks the end of embryogenesis and the beginning of the larval stage (Gilbert, 2017). The reported period for normal hatching of developing embryos is between 48 and 72 hpf (Kimmel et al., 1995). Therefore, even if the hatch occurs in a time interval considered normal, we found that there was an increase in the number of hatches in the exposed animals, which highlights the interference of the contaminant. Whereas hatching occurs due to the activity of proteolytic enzymes in specialized cells, hormonal metabolic patterns and the movements of muscle contractions performed by the embryo (Samaee et al., 2015), this increase in hatching rates, during this period, may probably be due to alterations in proteolytic enzymes. Among the candidates we point out the enzyme responsible for breaking the chorionic barrier, the incubation 1 enzyme (HE1) (Sano et al., 2008).

Regarding the decrease in heart rate of 48 hpf, we showed that it occurred in animals exposed to concentrations between 2mg/L and 100 mg/L of glyphosate. Different studies found a decrease in heart rate of embryos exposed to glyphosate in a concentration dependent manner (Gaur and Bhargava, 2019; Lanzarin et al., 2019). It is known that the heart rate of the zebrafish ranges from 120 to 180 beats per minute (bpm) during the early stages of development (Bournele and Beis, 2016). It became clear that at lower concentrations, there is a greater likelihood of seeing an effect on heart rate. This decrease may be associated with genes involved in heart contraction and excitation *Cacna1C (Calcium Voltage-Gated Channel Subunit Alpha1 C)* and *ryr2a* (*Ryanodine receptor 2a*), altering calcium homeostasis through the expression of *Heat Shock Protein Family B (Small) Member 11* (hspb11) involved in the signaling pathway of nitric oxide (NO) (Gaur and Bhargava, 2019). In addition, another reason may be that the

presence of acetylcholinesterase (AChE) inhibitors, which therefore causes an increase in the concentration of acetylcholine in the synaptic cleft, causing continuous acetylcholine receptor stimulation and decreasing heart rate (Lin et al., 2007).

The zebrafish's cardiac pumping system goes through several essential processes of functional maturation and, once damages occur during development at this stage, they result in congenital cardiac abnormalities (Beis et al., 2005). Associated with these results, we found an increase in mortality in animals exposed to glyphosate at 72 hpf. There is evidence showing an increase in mortality in embryos with 72 hpf at concentrations of 8.5mg/L and 15 mg/L of commercial glyphosate (Lanzarin et al., 2019). In our review, we found that the decrease in heart rate persisted in larvae at 72 hpf. These results are justified by the prevalence of structural abnormalities caused by glyphosate. This occurred both in the atrium and in the ventricle, with the presence of rupture of the cardiac wall, leading to functional impairment of pumping (Lanzarin et al., 2019; Roy et al., 2016b; Yusof et al., 2014).

Adequate cardiac function depends on high energy conversion efficiency (Frederik et al., 2007). Thus, due to the edema found in the yolk sac, the normal functioning of the heart is strongly affected, leading to a decrease in heart rate by reducing the local energy supply (Raldúa et al., 2008). These damages to the heart lead to systemic pathophysiological implications, affect the availability of oxygen for the basal cell metabolism and impair the blood transport itself, affecting energy production, fundamental to the development of the embryo (Marieb e Hoehn, 2001).

There is a relationship between glyphosate accelerating the hatching of embryos by 72 hpf and the metabolic damage caused by cardiac system failure. We observed in our work that the higher the concentration of glyphosate used, the greater the chances of these effects leading to an outcome of increased hatching and mortality. Hatch rate is a sensitive parameter to assess the interference of chemicals in embryo development (OCDE, 2013). Thus, embryos can become more vulnerable to predatory attacks, mechanical and osmotic stress and chemical compounds present in the external environment, making changes at this stage potentially lethal (Kimmel et al., 1995; Samaee et al., 2015).

At 96 hpf we only verified that glyphosate increased mortality in animals in contact with the contaminant. However, different studies found that, for this same period, the larvae had lower body length than expected, head and eyes reduction (Zhang et al., 2017). There was also a decrease in carbonic anhydrase activity (EC 4.2.1.1) and hexokinase (EC 2.7.1.1), and the increase in apoptotic response (Panetto et al., 2019; Sulukan et al., 2017).

Unlike larvae, in adult specimens it was possible to analyze the specific effects of the organ from the methodological peculiarity of the assessment of biochemical responses. Thus, we found that animals exposed to glyphosate showed an increase in reactive oxygen species in the gills (exposed for 24h) and an increase in antioxidant capacity against *peroxyl* radicals in the gills (exposed for 96h). Adult fish show changes in brain thiol levels, as well as increased lipid peroxidation markers in brain, liver and muscle (Lopes et al., 2018; Santo et al., 2018). The activities of enzymes *catalase* (EC 1.11.1.6) and *glutationa peroxidase* (EC 1.11.1.9) showed affected activities in the liver, increase in ACAP (antioxidant capacity against peroxyl radicals) and reduction in ROS (reactive oxygen species) in the gills and in the liver (Santo et al., 2018; Velasques et al., 2016). Thus, these enzymatic alterations reduce cellular defenses, increasing the susceptibility to toxic xenobiotic substances, which under normal conditions would be metabolized, as well as impairing gas exchange and osmoregulation.

As for the effects related to the behavior of the species, we observed a decrease in distance traveled and mean speed, in addition to an increase in the number of rotations. We verified that there was a relationship between the increase in glyphosate concentration and the presence of disorders associated with behavior. Adult animals exposed to glyphosate spend more time in the upper zone and less time in the lower part of the test field. Rotational behavior was increased, they spend less time in the treated range, impaired memory and reduced aggressive behavior (Bridi et al., 2017; da Costa Chaulet et al., 2019; da Rosa et al., 2016).

The significant changes found in this review, supported by several studies, demonstrate the potential toxicity of this herbicide for fish populations that live in a contaminated environment. Our data raise important concerns about potential harm to wild fish in the embryonic, larval and adult stages. That said, the data show that by affecting the embryonic stage, the chances of mortality increase, or else, complications that will remain throughout the life of the animal. The resulting impacts can compromise the search for food, the local reproduction and perpetuation of the species and the socialization itself.

5. Conclusion

We conclude that pure glyphosate and glyphosate-based herbicide formulations can be considered toxic for the development of the *Danio rerio* species. Since, in the embryonic and larval stages, they induce an increase in mortality, cause malformations in the body, affect hatching and heart rate. At high concentrations, we found a greater probability of observing an

effect on morphology, hatching, malformations and mortality. In addition, we observed biochemical and behavioral damage caused in the adult stage of this fish.

We believe that our findings indicate damage caused mainly in the embryonic and larval stages of this fish species. Due to lethal effects on animals, or sublethal effects that cause difficulties in predation, feeding, locomotion, reproduction and survival, this and/or other fish species can be extinct. Therefore, precautionary and damage mitigation measures must be taken to reduce the potential risks of glyphosate to fish and other aquatic species. Thus, our study reinforces the necessary and urgent attention to the risks of glyphosate and its commercial formulations for the health of fish and for the entire aquatic system.

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Figures legend

Figure 1. Flowchart of the selected articles in this systematic review and meta-analysis study.

Figure 2. Risk of bias assessment in the selected articles.

Figure 3. Forest plot of odds ratios (OR) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on mortality in embryo and larva zebrafish. We considered a sub-group analysis based on the hours post fertilization.

Figure 4. Forest plot of odds ratios (OR) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on hatching in embryo and larva zebrafish. We considered a sub-group analysis based on the hours post fertilization.

Figure 5. Forest plot of odds ratios (OR) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on malformation in embryo and larva zebrafish. We considered a sub-group analysis based on the structure of body.

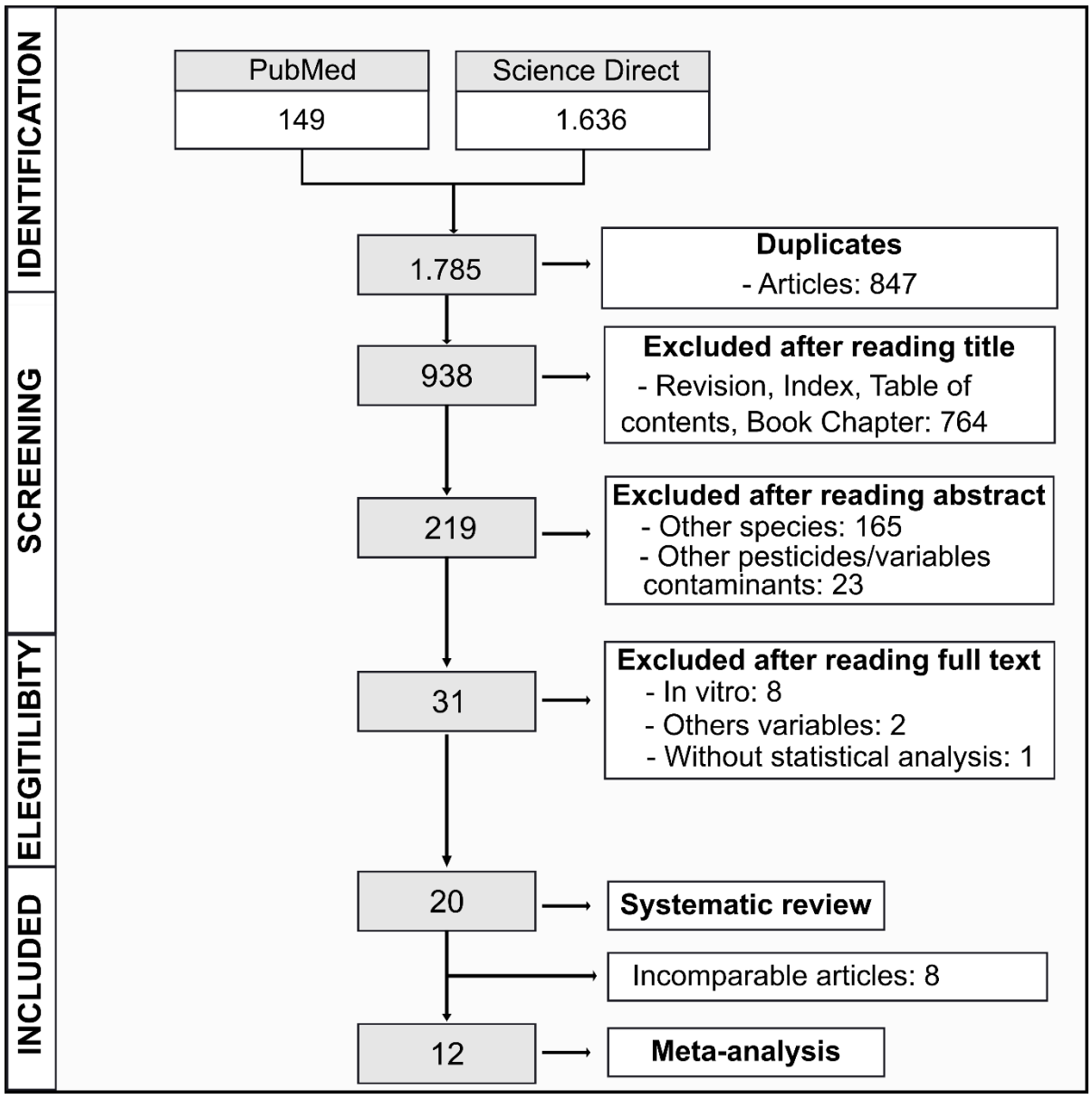
Figure 6. Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on morphology in embryo and larva zebrafish. We considered a sub-group analysis based on the structure of body.

Figure 7. Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on heart rate in embryo and larva zebrafish. We considered a sub-group analysis based on the hours post fertilization.

Figure 8. Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on biochemistry in adult zebrafish. We considered a sub-group analysis based on the biomarkers.

Figure 9. Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CIs) for the effect of glyphosate concentration on behavior in adult zebrafish. We considered a sub-group analysis based on the type of behavior.

Figures



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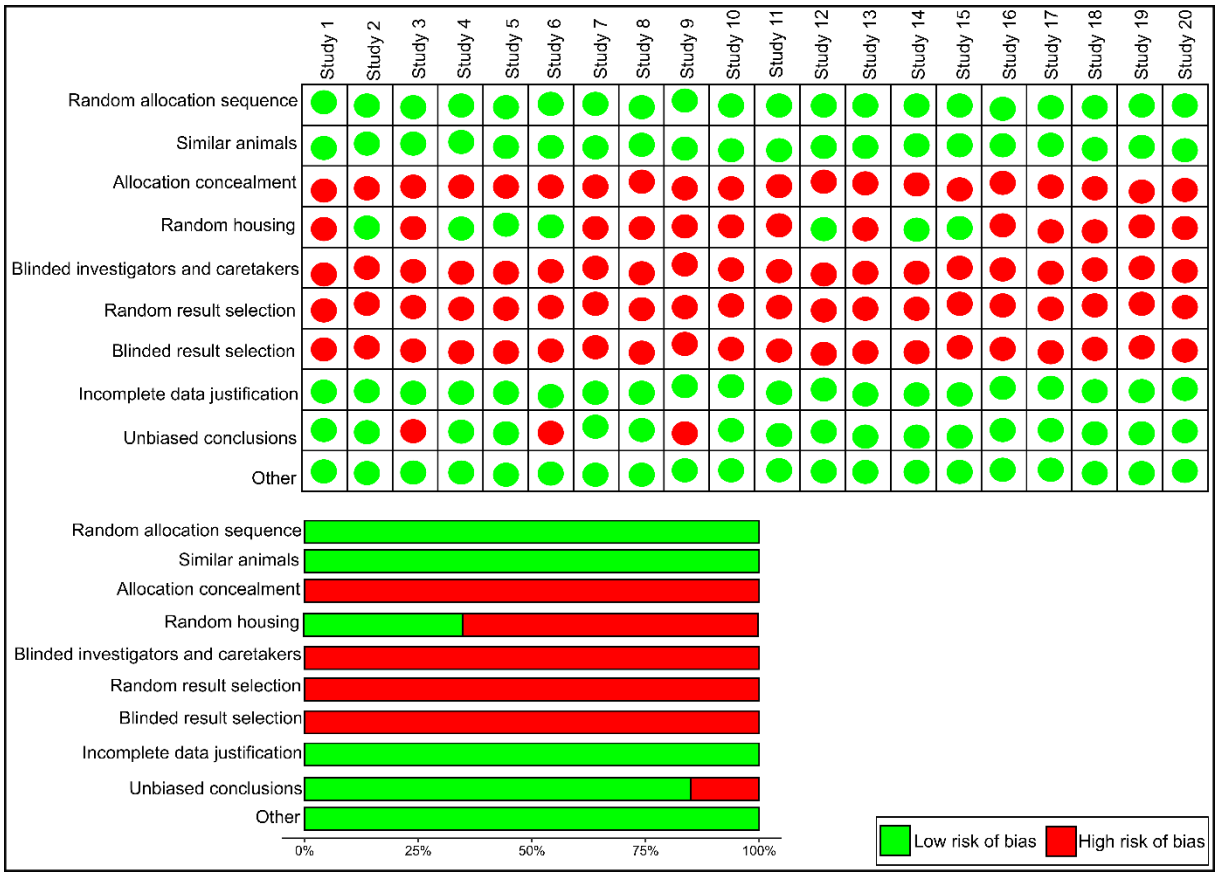
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Study 2
Study 3
Study 4
Study 5
Study 6
Study 7
Study 8
Study 9
Study 10
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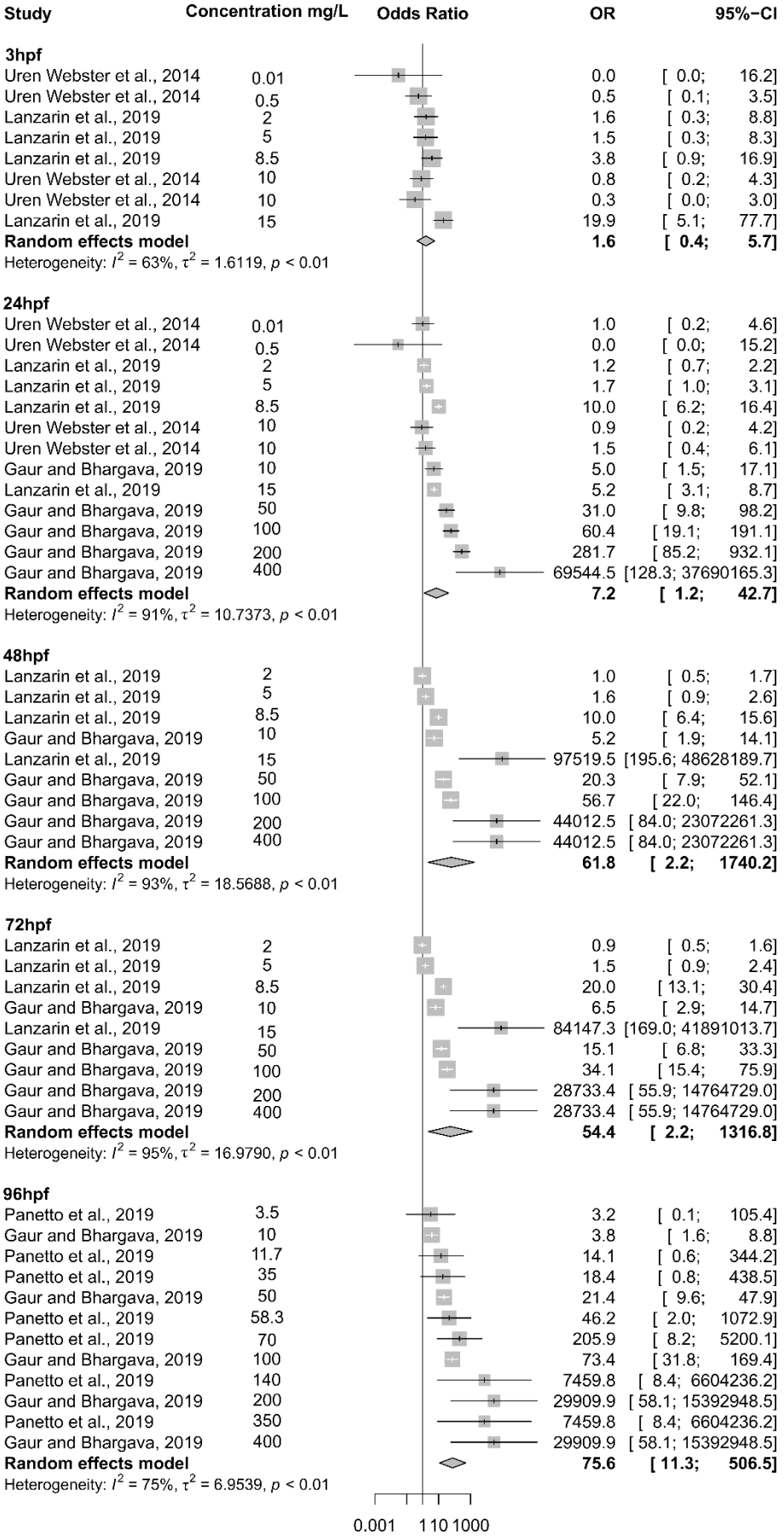
Study 12
Study 13
Study 14
Study 15
Study 16
Study 17
Study 18
Study 19
Study 20
Random allocation sequence
Similar animals
Allocation concealment
Random housing
Blinded investigators and caretakers
Random result selection
Blinded result selection
Incomplete data justification
Unbiased conclusions
Other
0%
25%
50%
75%
100%
Low risk of bias
High risk of bias

Figure 2.


Study
Concentration mg/L
Odds Ratio
OR
95%−CI
3hpf
Uren Webster et al., 2014 0.01 0.0 [0.0; 16.2]
Uren Webster et al., 2014 0.5 0.5 [0.1; 3.5]
Lanzarin et al., 2019 2 1.6 [0.3; 8.8]
Lanzarin et al., 2019 5 1.5 [0.3; 8.3]
Lanzarin et al., 2019 8.5 3.8 [0.9; 16.9]
Uren Webster et al., 2014 10 0.8 [0.2; 4.3]
Uren Webster et al., 2014 10 0.3 [0.0; 3.0]
Lanzarin et al., 2019 15 19.9 [5.1; 77.7]
Random effects model 1.6 [0.4; 5.7]
Heterogeneity: I^2 = 63%, τ^2 = 1.6119, p < 0.01
24hpf
Uren Webster et al., 2014 0.01 1.0 [0.2; 4.6]
Uren Webster et al., 2014 0.5 0.0 [0.0; 15.2]
Lanzarin et al., 2019 2 1.2 [0.7; 2.2]
Lanzarin et al., 2019 5 1.7 [1.0; 3.1]
Lanzarin et al., 2019 8.5 10.0 [6.2; 16.4]
Uren Webster et al., 2014 10 0.9 [0.2; 4.2]
Uren Webster et al., 2014 10 1.5 [0.4; 6.1]
Gaur and Bhargava, 2019 10 5.0 [1.5; 17.1]
Lanzarin et al., 2019 15 5.2 [3.1; 8.7]
Gaur and Bhargava, 2019 50 31.0 [9.8; 98.2]
Gaur and Bhargava, 2019 100 60.4 [19.1; 191.1]
Gaur and Bhargava, 2019 200 281.7 [85.2; 932.1]
Gaur and Bhargava, 2019 400 69544.5 [128.3; 37690165.3]
Random effects model 7.2 [1.2; 42.7]
Heterogeneity: I^2 = 91%, τ^2 = 10.7373, p < 0.01
48hpf
Lanzarin et al., 2019 2 1.0 [0.5; 1.7]
Lanzarin et al., 2019 5 1.6 [0.9; 2.6]
Lanzarin et al., 2019 8.5 10.0 [6.4; 15.6]
Gaur and Bhargava, 2019 10 5.2 [1.9; 14.1]
Lanzarin et al., 2019 15 97519.5 [195.6; 48628189.7]
Gaur and Bhargava, 2019 50 20.3 [7.9; 52.1]
Gaur and Bhargava, 2019 100 56.7 [22.0; 146.4]
Gaur and Bhargava, 2019 200 44012.5 [84.0; 23072261.3]
Gaur and Bhargava, 2019 400 44012.5 [84.0; 23072261.3]
Random effects model 61.8 [2.2; 1740.2]
Heterogeneity: I^2 = 93%, τ^2 = 18.5688, p < 0.01
72hpf
Lanzarin et al., 2019 2 0.9 [0.5; 1.6]
Lanzarin et al., 2019 5 1.5 [0.9; 2.4]
Lanzarin et al., 2019 8.5 20.0 [13.1; 30.4]
Gaur and Bhargava, 2019 10 6.5 [2.9; 14.7]
Lanzarin et al., 2019 15 84147.3 [169.0; 41891013.7]
Gaur and Bhargava, 2019 50 15.1 [6.8; 33.3]
Gaur and Bhargava, 2019 100 34.1 [15.4; 75.9]
Gaur and Bhargava, 2019 200 28733.4 [55.9; 14764729.0]
Gaur and Bhargava, 2019 400 28733.4 [55.9; 14764729.0]
Random effects model 54.4 [2.2; 1316.8]
Heterogeneity: I^2 = 95%, τ^2 = 16.9790, p < 0.01
96hpf
Panetto et al., 2019 3.5 3.2 [0.1; 105.4]
Gaur and Bhargava, 2019 10 3.8 [1.6; 8.8]
Panetto et al., 2019 11.7 14.1 [0.6; 344.2]
Panetto et al., 2019 35 18.4 [0.8; 438.5]
Gaur and Bhargava, 2019 50 21.4 [9.6; 47.9]
Panetto et al., 2019 58.3 46.2 [2.0; 1072.9]
Panetto et al., 2019 70 205.9 [8.2; 5200.1]
Gaur and Bhargava, 2019 100 73.4 [31.8; 169.4]
Panetto et al., 2019 140 7459.8 [8.4; 6604236.2]
Gaur and Bhargava, 2019 200 29909.9 [58.1; 15392948.5]
Panetto et al., 2019 350 7459.8 [8.4; 6604236.2]
Gaur and Bhargava, 2019 400 29909.9 [58.1; 15392948.5]
Random effects model 75.6 [11.3; 506.5]
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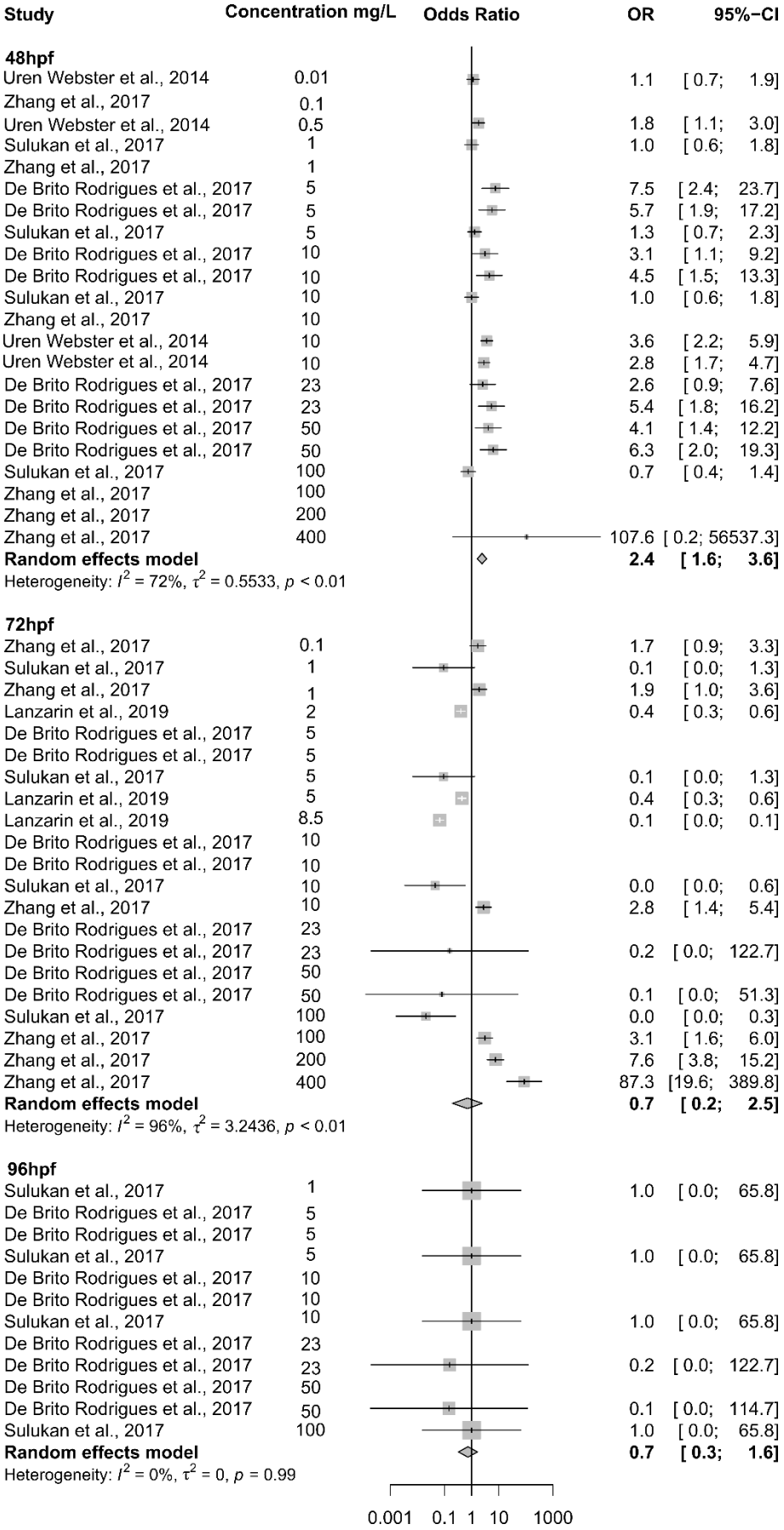
0.001
1
10
1000

Figure 3.


Study
Concentration mg/L
Odds Ratio
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48hpf
Uren Webster et al., 2014 0.01 1.1 [0.7; 1.9]
Zhang et al., 2017 0.1
Uren Webster et al., 2014 0.5 1.8 [1.1; 3.0]
Sulukan et al., 2017 1 1.0 [0.6; 1.8]
Zhang et al., 2017 1
De Brito Rodrigues et al., 2017 5 7.5 [2.4; 23.7]
De Brito Rodrigues et al., 2017 5 5.7 [1.9; 17.2]
Sulukan et al., 2017 5 1.3 [0.7; 2.3]
De Brito Rodrigues et al., 2017 10 3.1 [1.1; 9.2]
De Brito Rodrigues et al., 2017 10 4.5 [1.5; 13.3]
Sulukan et al., 2017 10 1.0 [0.6; 1.8]
Zhang et al., 2017 10
Uren Webster et al., 2014 10 3.6 [2.2; 5.9]
Uren Webster et al., 2014 10 2.8 [1.7; 4.7]
De Brito Rodrigues et al., 2017 23 2.6 [0.9; 7.6]
De Brito Rodrigues et al., 2017 23 5.4 [1.8; 16.2]
De Brito Rodrigues et al., 2017 50 4.1 [1.4; 12.2]
De Brito Rodrigues et al., 2017 50 6.3 [2.0; 19.3]
Sulukan et al., 2017 100 0.7 [0.4; 1.4]
Zhang et al., 2017 100
Zhang et al., 2017 200
Zhang et al., 2017 400 107.6 [0.2; 56537.3]
Random effects model 2.4 [1.6; 3.6]
Heterogeneity: I^2 = 72%, τ^2 = 0.5533, p < 0.01
72hpf
Zhang et al., 2017 0.1 1.7 [0.9; 3.3]
Sulukan et al., 2017 1 0.1 [0.0; 1.3]
Zhang et al., 2017 1 1.9 [1.0; 3.6]
Lanzarin et al., 2019 2 0.4 [0.3; 0.6]
De Brito Rodrigues et al., 2017 5
De Brito Rodrigues et al., 2017 5
Sulukan et al., 2017 5 0.1 [0.0; 1.3]
Lanzarin et al., 2019 5 0.4 [0.3; 0.6]
Lanzarin et al., 2019 8.5 0.1 [0.0; 0.1]
De Brito Rodrigues et al., 2017 10
De Brito Rodrigues et al., 2017 10
Sulukan et al., 2017 10 0.0 [0.0; 0.6]
Zhang et al., 2017 10 2.8 [1.4; 5.4]
De Brito Rodrigues et al., 2017 23
De Brito Rodrigues et al., 2017 23 0.2 [0.0; 122.7]
De Brito Rodrigues et al., 2017 50
De Brito Rodrigues et al., 2017 50 0.1 [0.0; 51.3]
Sulukan et al., 2017 100 0.0 [0.0; 0.3]
Zhang et al., 2017 100 3.1 [1.6; 6.0]
Zhang et al., 2017 200 7.6 [3.8; 15.2]
Zhang et al., 2017 400 87.3 [19.6; 389.8]
Random effects model 0.7 [0.2; 2.5]
Heterogeneity: I^2 = 96%, τ^2 = 3.2436, p < 0.01
96hpf
Sulukan et al., 2017 1 1.0 [0.0; 65.8]
De Brito Rodrigues et al., 2017 5
De Brito Rodrigues et al., 2017 5
Sulukan et al., 2017 5 1.0 [0.0; 65.8]
De Brito Rodrigues et al., 2017 10
De Brito Rodrigues et al., 2017 10
Sulukan et al., 2017 10 1.0 [0.0; 65.8]
De Brito Rodrigues et al., 2017 23
De Brito Rodrigues et al., 2017 23 0.2 [0.0; 122.7]
De Brito Rodrigues et al., 2017 50
De Brito Rodrigues et al., 2017 50 0.1 [0.0; 114.7]
Sulukan et al., 2017 100 1.0 [0.0; 65.8]
Random effects model 0.7 [0.3; 1.6]
Heterogeneity: I^2 = 0%, τ^2 = 0, p = 0.99
0.001 0.1 1 10 1000

Figure 4.

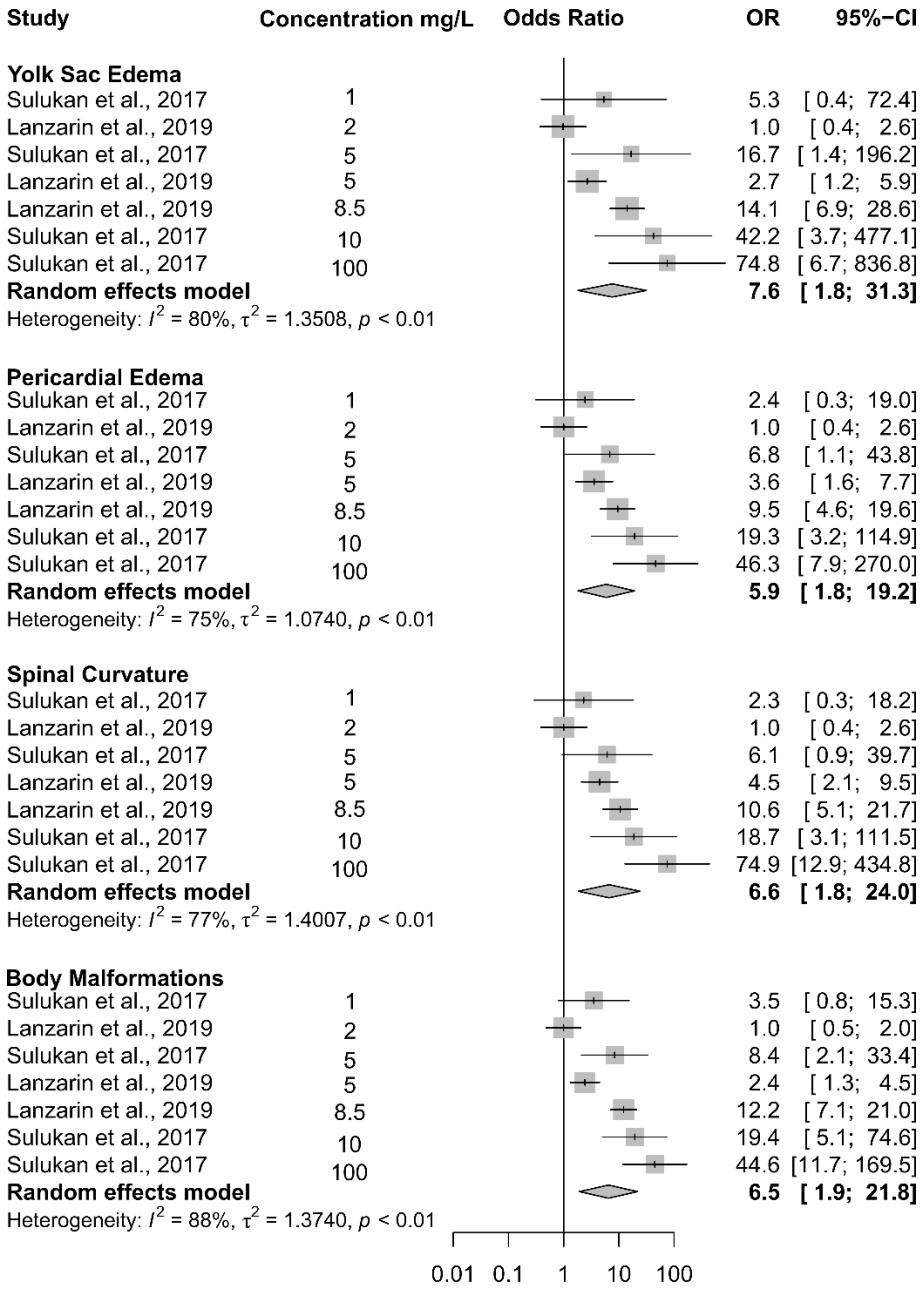
Study
Concentration mg/L
Odds Ratio
OR
95%−CI
Yolk Sac Edema
Sulukan et al., 2017 1 5.3 [0.4; 72.4]
Lanzarin et al., 2019 2 1.0 [0.4; 2.6]
Sulukan et al., 2017 5 16.7 [1.4; 196.2]
Lanzarin et al., 2019 5 2.7 [1.2; 5.9]
Lanzarin et al., 2019 8.5 14.1 [6.9; 28.6]
Sulukan et al., 2017 10 42.2 [3.7; 477.1]
Sulukan et al., 2017 100 74.8 [6.7; 836.8]
Random effects model 7.6 [1.8; 31.3]
Heterogeneity: I^2 = 80%, τ^2 = 1.3508, p < 0.01
Pericardial Edema
Sulukan et al., 2017 1 2.4 [0.3; 19.0]
Lanzarin et al., 2019 2 1.0 [0.4; 2.6]
Sulukan et al., 2017 5 6.8 [1.1; 43.8]
Lanzarin et al., 2019 5 3.6 [1.6; 7.7]
Lanzarin et al., 2019 8.5 9.5 [4.6; 19.6]
Sulukan et al., 2017 10 19.3 [3.2; 114.9]
Sulukan et al., 2017 100 46.3 [7.9; 270.0]
Random effects model 5.9 [1.8; 19.2]
Heterogeneity: I^2 = 75%, τ^2 = 1.0740, p < 0.01
Spinal Curvature
Sulukan et al., 2017 1 2.3 [0.3; 18.2]
Lanzarin et al., 2019 2 1.0 [0.4; 2.6]
Sulukan et al., 2017 5 6.1 [0.9; 39.7]
Lanzarin et al., 2019 5 4.5 [2.1; 9.5]
Lanzarin et al., 2019 8.5 10.6 [5.1; 21.7]
Sulukan et al., 2017 10 18.7 [3.1; 111.5]
Sulukan et al., 2017 100 74.9 [12.9; 434.8]
Random effects model 6.6 [1.8; 24.0]
Heterogeneity: I^2 = 77%, τ^2 = 1.4007, p < 0.01
Body Malformations
Sulukan et al., 2017 1 3.5 [0.8; 15.3]
Lanzarin et al., 2019 2 1.0 [0.5; 2.0]
Sulukan et al., 2017 5 8.4 [2.1; 33.4]
Lanzarin et al., 2019 5 2.4 [1.3; 4.5]
Lanzarin et al., 2019 8.5 12.2 [7.1; 21.0]
Sulukan et al., 2017 10 19.4 [5.1; 74.6]
Sulukan et al., 2017 100 44.6 [11.7; 169.5]
Random effects model 6.5 [1.9; 21.8]
Heterogeneity: I^2 = 88%, τ^2 = 1.3740, p < 0.01
0.01 0.1 1 10 100

Figure 5.

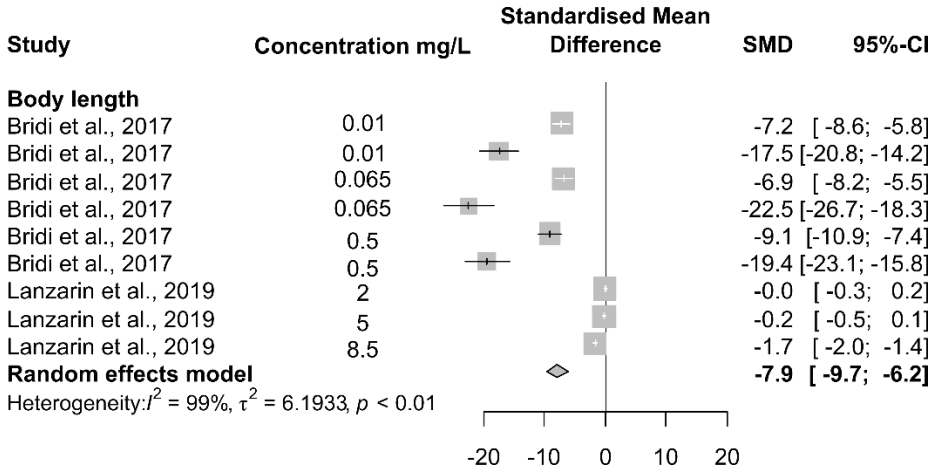

Figure 6.

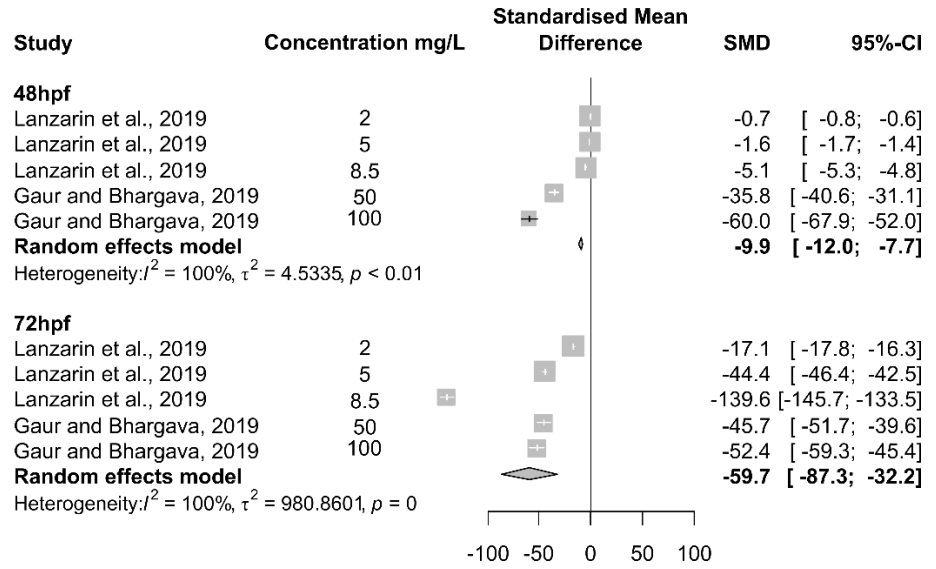

Figure 7.

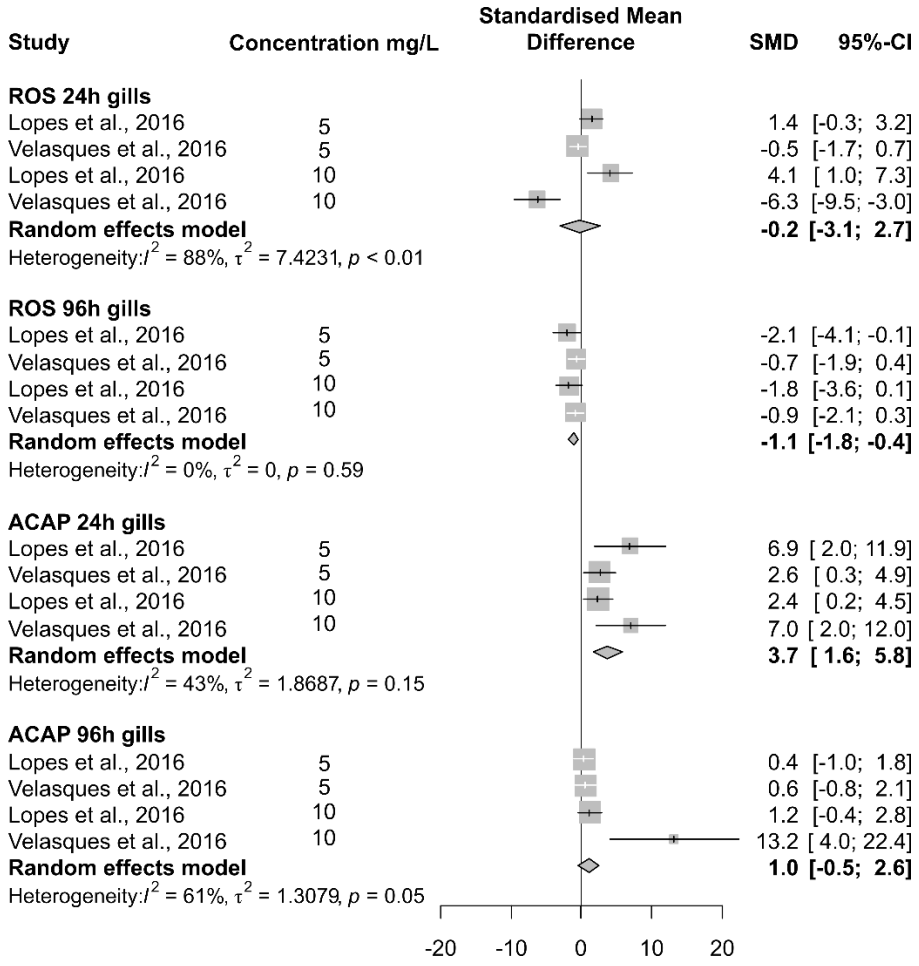
Study
Concentration mg/L
Standardised Mean Difference
SMD
95%-CI
ROS 24h gills
Lopes et al., 2016 5 1.4 [-0.3; 3.2]
Velasques et al., 2016 5 -0.5 [-1.7; 0.7]
Lopes et al., 2016 10 4.1 [1.0; 7.3]
Velasques et al., 2016 10 -6.3 [-9.5; -3.0]
Random effects model -0.2 [-3.1; 2.7]
Heterogeneity: I^2 = 88%, τ^2 = 7.4231, p < 0.01
ROS 96h gills
Lopes et al., 2016 5 -2.1 [-4.1; -0.1]
Velasques et al., 2016 5 -0.7 [-1.9; 0.4]
Lopes et al., 2016 10 -1.8 [-3.6; 0.1]
Velasques et al., 2016 10 -0.9 [-2.1; 0.3]
Random effects model -1.1 [-1.8; -0.4]
Heterogeneity: I^2 = 0%, τ^2 = 0, p = 0.59
ACAP 24h gills
Lopes et al., 2016 5 6.9 [2.0; 11.9]
Velasques et al., 2016 5 2.6 [0.3; 4.9]
Lopes et al., 2016 10 2.4 [0.2; 4.5]
Velasques et al., 2016 10 7.0 [2.0; 12.0]
Random effects model 3.7 [1.6; 5.8]
Heterogeneity: I^2 = 43%, τ^2 = 1.8687, p = 0.15
ACAP 96h gills
Lopes et al., 2016 5 0.4 [-1.0; 1.8]
Velasques et al., 2016 5 0.6 [-0.8; 2.1]
Lopes et al., 2016 10 1.2 [-0.4; 2.8]
Velasques et al., 2016 10 13.2 [4.0; 22.4]
Random effects model 1.0 [-0.5; 2.6]
Heterogeneity: I^2 = 61%, τ^2 = 1.3079, p = 0.05
-20
-10
0
10
20

Figure 8.

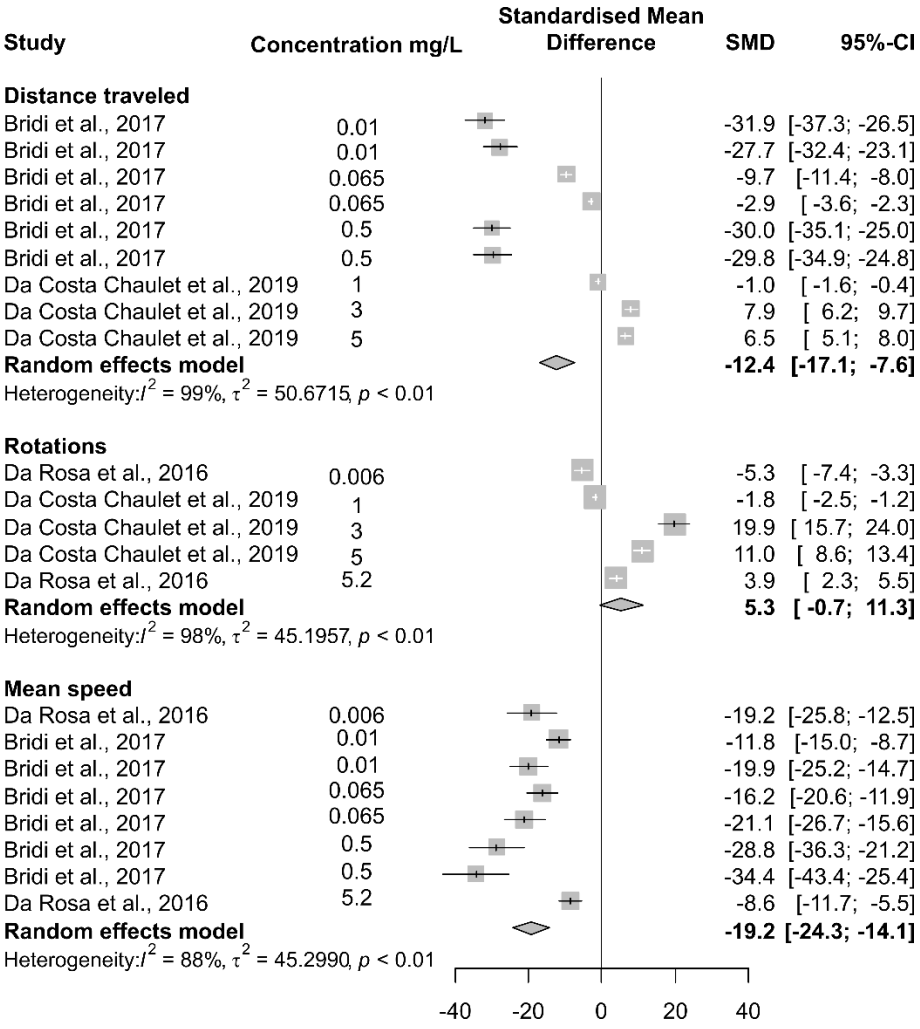
Study
Concentration mg/L
Standardised Mean Difference
SMD
95%-CI
Distance traveled
Bridi et al., 2017 0.01 -31.9 [-37.3; -26.5]
Bridi et al., 2017 0.01 -27.7 [-32.4; -23.1]
Bridi et al., 2017 0.065 -9.7 [-11.4; -8.0]
Bridi et al., 2017 0.065 -2.9 [-3.6; -2.3]
Bridi et al., 2017 0.5 -30.0 [-35.1; -25.0]
Bridi et al., 2017 0.5 -29.8 [-34.9; -24.8]
Da Costa Chaulet et al., 2019 1 -1.0 [-1.6; -0.4]
Da Costa Chaulet et al., 2019 3 7.9 [6.2; 9.7]
Da Costa Chaulet et al., 2019 5 6.5 [5.1; 8.0]
Random effects model -12.4 [-17.1; -7.6]
Heterogeneity: I^2 = 99%, τ^2 = 50.6715, p < 0.01
Rotations
Da Rosa et al., 2016 0.006 -5.3 [-7.4; -3.3]
Da Costa Chaulet et al., 2019 1 -1.8 [-2.5; -1.2]
Da Costa Chaulet et al., 2019 3 19.9 [15.7; 24.0]
Da Costa Chaulet et al., 2019 5 11.0 [8.6; 13.4]
Da Rosa et al., 2016 5.2 3.9 [2.3; 5.5]
Random effects model 5.3 [-0.7; 11.3]
Heterogeneity: I^2 = 98%, τ^2 = 45.1957, p < 0.01
Mean speed
Da Rosa et al., 2016 0.006 -19.2 [-25.8; -12.5]
Bridi et al., 2017 0.01 -11.8 [-15.0; -8.7]
Bridi et al., 2017 0.01 -19.9 [-25.2; -14.7]
Bridi et al., 2017 0.065 -16.2 [-20.6; -11.9]
Bridi et al., 2017 0.065 -21.1 [-26.7; -15.6]
Bridi et al., 2017 0.5 -28.8 [-36.3; -21.2]
Bridi et al., 2017 0.5 -34.4 [-43.4; -25.4]
Da Rosa et al., 2016 5.2 -8.6 [-11.7; -5.5]
Random effects model -19.2 [-24.3; -14.1]
Heterogeneity: I^2 = 88%, τ^2 = 45.2990, p < 0.01
-40
-20
0
20
40

Figure 9.

Tables

Table 1: Studies description.

Authors	Year	Life Cycle	Glyphosate	Concentration mg/L	Dilution	Reposition	Exposure time (h)	Temperature ºC	Analysis	Main results
Bridi et al	2017	embryo, larval and adult	Pure and Commercial	0.01, 0.065 and 0.5	System water	No	96	28	Survival at 24, 48 and 72 hpf; Morphological parameters (body length, ocular distance, surface area); Exploratory behavior of larvae; Bouncing-ball avoidance behavior of larvae; Adult exploratory behavior; Adult aggressive behavior; and Aversive memory in adults.	In larvae there was a decrease in ocular distance exposed to 0.5 mg / L of pure glyphosate and differences in locomotor activity and aversive behavior after exposure to pure or commercial glyphosate. In adults exposed to pure glyphosate at 0.5 mg/L and commercial glyphosate at 0.065 or 0.5 mg/L, reduced distance traveled, average speed and line passes. Also, in adults, commercial glyphosate at 0.5 mg/L caused significant memory impairment. In addition, all pure and commercial glyphosate concentrations reduced aggressive behavior
Lanzarin et al	2019	embryo and larval	Commercial	2, 5, 8.5 and 15	System water	Yes	72	28	Mortality at 3, 6, 22, 46 and 72h; Hatching rate at 72 h; Heart rate at 46 and 72 h; Morphological abnormalities (body length, eye diameter, brain structures); Histological analysis; and Biochemical and antioxidant markers (ROS, SOD, CAT, GST, GSH, GSSG, OSI, LPO, AChE and LDH).	Increased mortality was observed in embryos exposed to concentrations above 8.5 mg a.i. mL^{1}, as well as an increase in the number of malformations. Decreased heart rate and hatchability were also observed. The concentrations induced a dose-dependent decrease in heart rate while not inducing significant developmental changes.
Gaur and Bhargava	2019	embryo and larval	Pure	10, 50, 100, 200 and 400	E3 embryo medium	Yes	96	28	Mortality at 24, 48, 72 and 96 h; Morphological abnormalities; Heart beat at 48 and 72 h; Gene expression (Cacna1C, Ryr2a, hspb11); and Nitric oxide generation	Glyphosate significantly reduced heart rate in a time and concentration-dependent manner indicating cardiotoxicity. Selective down-regulation of Cacana1C and ryr2a genes, along with selective up-regulation of the hspb11 gene was observed after exposure to glyphosate. Reduction in the generation of nitric oxide was also observed.
Uren Webster et al	2014	embryo and larval	Pure and Commercial	Pure: 10; Commercial: 0.01, 0.5 and 10	System water plus methylene blue	No	72	28	Egg production; Mortality at 3.5 and hpf; Hatched 54 hpf; Morphometric Parameters (mass and legth); and Gonad Transcript Profiling (cat, gstp1, sod1, gpx1a, baxa, tp53, cyp19a1, cyp17a1, cyp11a1, hsd3b2, esr2a, esr2b, esr1a, star and ar).	10 mg/L pure glyphosate reduced egg production but not fertilization rate. Exposure to 10 mg/L of pure, commercial glyphosate increased early-stage mortality and premature hatch. The commercial formulation caused alterations in the expression of cyp19a1 and esr1 in the ovary and hsd3b2, cat and sod1 in the testes.

Panetto et al	2019	embryo and larval	Commercial	3.5, 11.7, 35, 58.3, 70, 140 and 350	E3 embryo medium	No	96	28	Biochemical tests (Protein concentration, Triglyceride, Glucose, Glycogen, Hexokinase, Hexokinase, Aspartate aminotransferase, Alanine aminotransferase); Morphological alterations; and Molecular docking (glucokinase and hexokinases 1 and 2)	Hexokinase activity was significantly altered after exposure to 11.7 mg/L of commercial glyphosate. Glucokinase and hexokinases 1 and 2 interactions with glyphosate showed significant interactions in local activity. The present work demonstrates changes in swim bladder development and mortality. Protein, glucose, triglyceride and glycogen levels and alanine aminotransferase and aspartate aminotransferase activities were not significantly affected. However, hexokinase activity was significantly affected by exposure to 11.7 mg/L, 35 mg/mL and 58.3 mg/L commercial glyphosate.
De Brito Rodrigues et al	2017	embryo and larval	Commercial	5, 10, 23 and 50	System water	No	96	27	Mortality at 24, 48, 72 and 96 h; Genotoxicity; and Hatching rate 48, 72 and 96 h	The results showed that in the two commercial formulations used there was an increase in mortality at all times analyzed. Regarding hatching rate, one of the formulations increased the number of hatches in 48 h at all concentrations used, the other formulation only at the lowest dosage. Genotoxicity was not verified.
Sulukan et al	2017	embryo and larval	Commercial	1, 5, 10 and 100	E3 embryo medium	Yes	96	28	Morphological abnormalities; Biochemical tests (Carbonic anhydrase, Reactive oxygen species); Apoptosis detection; Survival rate; and Hatching rate at 24, 48, 72 and 96 hpf	The survival rate decreased dose-dependent; glyphosate delayed hatching at a concentration of 100 mg/L in the zebrafish embryo. There was significantly, even at the lowest dose in body malformations, although no significant difference was found in relation to other malformations at the concentration of 1 mg/L. The CA enzyme activity was dose-dependently inhibited in all treatment groups in in relation to control. It detected fluorescent signal from different parts of the body such as eye, head, muscle, and intestine, especially at the highest dose of treatment. Also, the treatment resulted in a dose-dependent increase in global cell death.
Zhang et al	2017	embryo and larval	Pure	0.1, 1, 10, 100, 200 and 400	System water	Yes	96	28	Suvival at 6, 10, 24, 48, 72 and 96 hpf; Hatching rate at 48 and 72 hpf; Morphological analyses; Locomotor activity; Surface tension; Gene expression (ntl, krox20, shh); and Immunofluorescence	There was delay in the epibolic process, it increased as glyphosate concentrations increased. From 100 to 400 mg/L, exposure resulted in shorter body lengths, smaller eyes and heads. Exposure to higher concentrations increased the hatch rate. The ntl expression pattern in treated embryos was lower, the krox20 expression was altered as the concentrations increased and the shh level was normal. It demonstrated that larvae exposed to glyphosate increased their locomotor activities, mainly 0.01 and 1 mg/L. With increasing concentrations of glyphosate, the motoneurons became smaller and smaller, mainly at 10 mg/L.

Da Costa Chaulet et al	2019	Adult	Commercial	1, 3 and 5	System water	No	96	27	Locomotor parameters (distance traveled, rotations, number of crossings, time spent in the top zone, latency to entry into the top zone, time spent in the bottom zone)	Zebrafish exposed to 3 and 5 mg / L of GBH spends more time in the top zone and less time in the bottom zone.
Da Rosa et al	2016	Adult	Commercial	0.006 and 5.2	System water	No	96	24	Locomotor parameters (Time spent, distance, mean speed, absolute turn angle, rotations)	The glyphosate-based herbicide was found to be aversive to fish.
Lopes et al	2016	Adult	Pure	5 and 10	System water	No	96	28	Biochemical tests (ROS, ACAP, Lipid peroxidation, AChE); Gene expression (acetylcholinesterase)	An increase in ACAP in the gills after 24 h was observed in animals exposed to 5mg/L of glyphosate. A decrease in LPO in the brain tissue of animals exposed to 10mg/L after 24 h, while an increase was observed in muscle after 96 h. Insignificant alterations were observed in the generation of ROS. AChE activity was not altered in the animals' muscles or brains exposed to any concentration of glyphosate for 24 or 96 h. However, the gene expression of this enzyme in the brain it was reduced after 24 hours and increased in both brain and muscle tissue after 96 hours.
Velasques et al	2016	adult	Commercial	5 and 10	System water	No	96	28	Biochemical tests (ROS, ACAP); Gene expression (nrf2, cat, sod1, sod2 gclc, gpx, gst, ucp1 and ucp3)	In the gills, an increase in ACAP was observed after 96 h in the greatest concentration. In the liver, a reduction in ROS and ACAP was observed after 24 h, while an increase in ACAP was observed after 48 h at the highest concentration. Exposure to the lowest concentration caused a reduction in ROS after 72 and 96 h. Regarding gene expression, a reduction in SOD2 and GSTp was observed. An increase in ucp1 expression was observed in the gills of animals exposed to the highest concentration after 24 h. GPX gene expression was reduced in the gills of animals exposed to the lowest concentration; however, it was induced in liver tissue after 96 h of exposure to the highest concentration.

Fiorino et al	2018	embryo and larval	Pure	0.005, 0.05, 5, 10 and 50	System water	Yes	120	26	Hatching rate at 72, 96 and 120 hpf; Malformations; Mortality at 48, 72, 96 and 120 hpf	There was an increase in mortality in embryos exposed to glyphosate at 48, 72, 96 and 120 hpf. Also, an increase in hatch rate at 72 and 96 hpf was observed. Significantly higher cumulative mortality was observed at the concentration of 50 mg/L. Furthermore, malformations were observed in animals exposed to glyphosate.
Lanzarin et al	2020	embryo and larval	Commercial	1, 2 and 5	System water	Yes	72	28	Mortality at 22, 46, 70 and 142 h; Exploratory behaviour (Speed, Distance to center, Absolute turn angle, Distance moved, Time active); Social behaviour (NND, IID); Response to stimuli; Cortisol assay	No significant changes were observed in relation to exploratory behavior in the standard open field. Anxiety-related behaviors were similar between groups, and no social interference was observed after exposure to these glyphosate concentrations. Likewise, cortisol levels remained similar between treatments. Even so, larvae exposed to 5 mg did not react to the presence of an aversive stimulus.
De Brito Rodrigues et al	2019	embryo and larval	Pure and Commercial	1.7, 5, 10, 23, 50 and 100	System water	No	96	26	Survival at 24, 48 and 72 hpf; Abnormalities; Comet assay.	In this research, malformations were found in animals exposed to glyphosate. Also, there was an increase in the DNA damage index at all concentrations.
Santo et al	2018	Adult	Commercial	5	System water	No	96	26	Biochemical parameters (total thiols, nonprotein thiols, lipid peroxidation, SOD, CAT, GPx); Genotoxicity assays	A decrease in the levels of total thiols in the gills was found, but in the liver it was not altered. In relation to non-protein thiols, no alterations were observed in these organs. There was an increase in lipid peroxidation in the gills and liver. Furthermore, when it came to changes in enzymes, an increase in catalase activity and a decrease in liver GPx was observed in exposed animals.
Moraes et al	2020	Adult	Pure and Commercial	0.1	System water	Yes	96	25	Biochemical parameters (ABCC proteins); Gene expression (abcc1, abcc2, abcc3, abcc4, abcc5, bac, b2m, ef1a and rpl13a)	Exposure to pure and commercial glyphosate increased ABCC protein activity and gene expression up to 3 times when compared to controls, indicating the activation of detoxification mechanisms. Only in the brain, exposure to commercial glyphosate did not stimulate the activity of ABCC proteins, nor did the expression of the abcc1 and abcc4 genes that responded to exposure to pure glyphosate.
Roy et al a	2016	embryo and larval	Pure and Commercial	50	E3 embryo medium	No	24	28	Morphology; Hybridization (pax2, pax6, otx2,ephA4, hoxb1a and krox-20); Immunohistochemistry	Morphological abnormalities were found in the exposed animals, including cephalic and ocular reductions and a loss of delineated cerebral ventricles. Concomitantly with structural changes in the developing brain, using in situ hybridization analysis, decreases in genes expressed in the eye, fore, and midbrain regions of the brain, including pax2, pax6,

										otx2 and ephA4 were detected. However, no changes were detected in the ephA4 hindbrain expression domains nor in the unique hindbrain markers krox-20 ehoxb1a. Furthermore, no changes in RA expression domains were detected in the hindbrain and spinal cord, but a loss of expression in the retina was detected.
Roy et al b	2016	embryo and larval	Pure	50	E3 embryo medium	No	48	28	Morphology; Heart beat; and Immunohistochemistry	Treatment with 50 g/ml of glyphosate resulted in structural abnormalities in the atrium and ventricle, irregular heart loop, situs inversus, as well as heartbeat. The vasculature of the body was also affected, and changes in the color patterns of Mef2/mef2ca
Jaramillo et al	2018	adult	Commercial	0.065, 0.65 and 6.5	System water	Yes	168	26	Gene expression (act, gapdh, ef1, b2m, rpl8, hprt1, hatn10, cat)	The expression stability of six reference genes and one expressed repetitive element in four organs of zebrafish exposed for 7 days demonstrated organ-specific expression variability. In the brain (male) and ovary (female), rpl8 was the most stable gene, whereas in the testis and gill of males, ef1α and β-act were the most stable genes, respectively.

Supplementary material

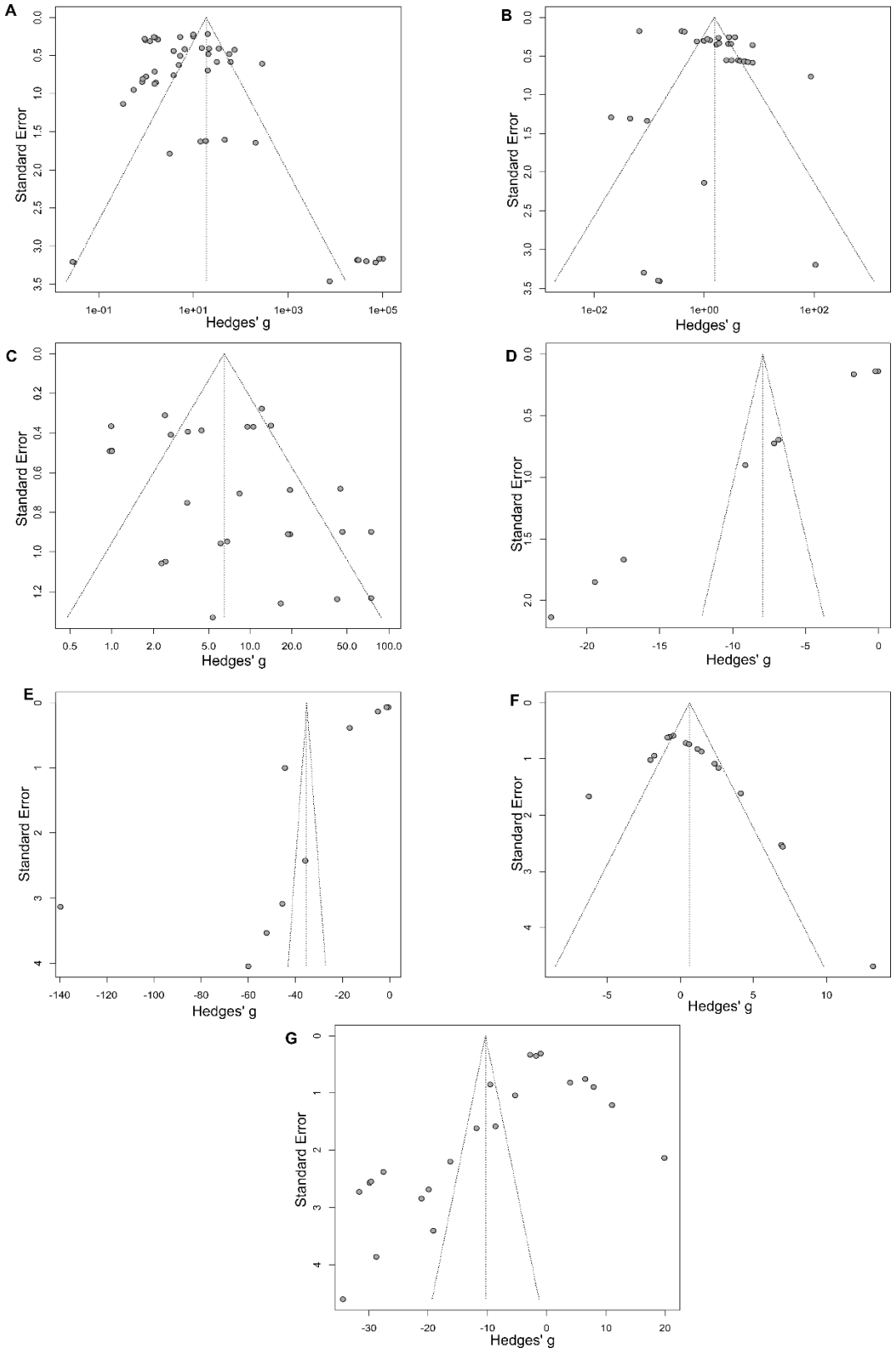

Figure S1. Funnel graph of effect of glyphosate in zebrafish on: (A) mortality in embryos and larvae; (B) hatching in embryos and larvae; (C) malformations in embryos and larvae; (D) morphology in embryos and larvae; (E) heart rate in embryos and larvae; (F) biochemistry in adult; (G) behavior in adult.

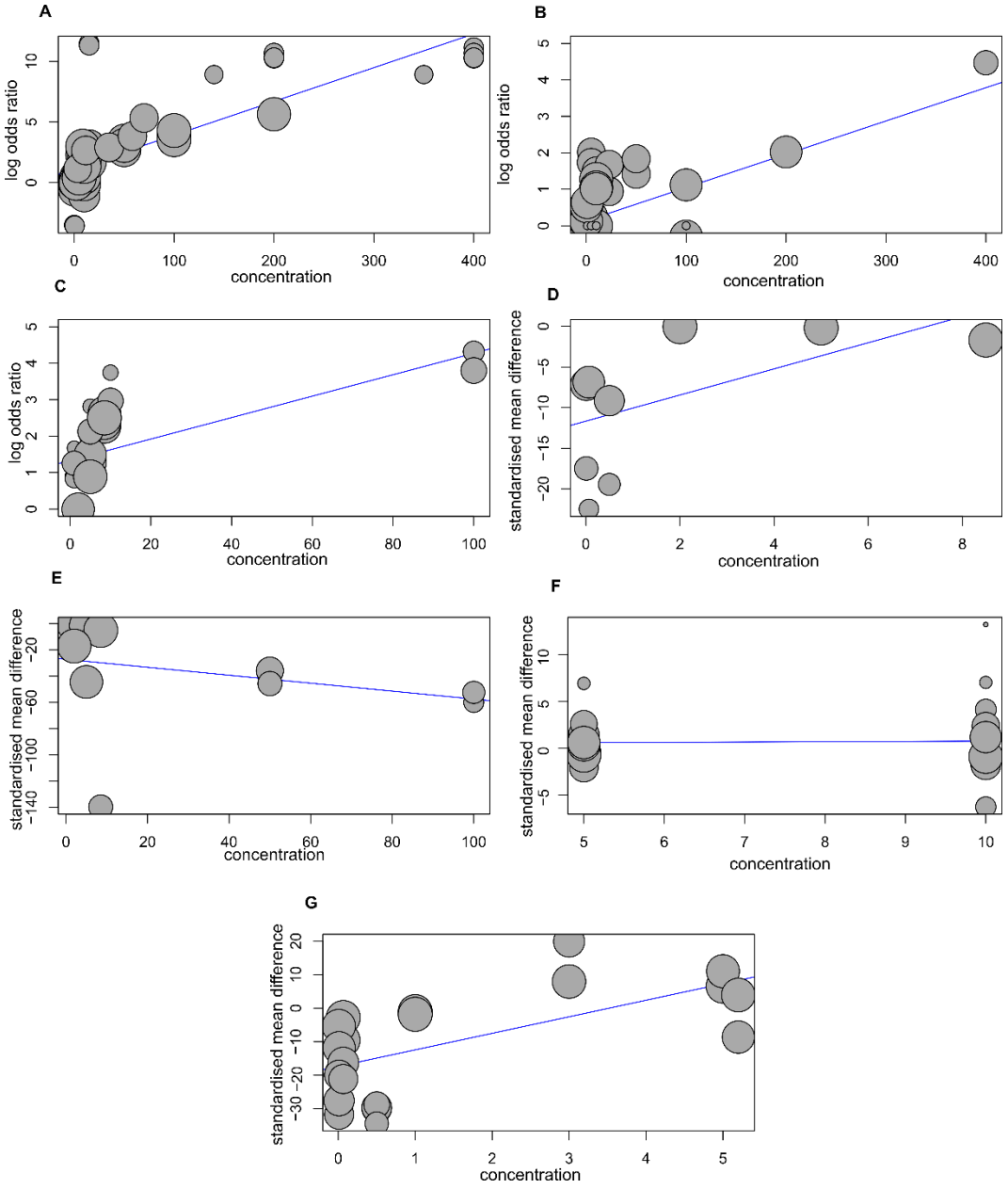

Figure S2. Meta-regression of the relationship between glyphosate concentration and the effect on zebrafish in: (A) mortality in embryos and larvae; (B) hatching in embryos and larvae; (C) malformations in embryos and larvae; (D) morphology in embryos and larvae; (E) heart rate in embryos and larvae; (F) biochemistry in adult; (G) behavior in adult.

6 DISCUSSÃO GERAL

A utilização de pesticidas vem aumentando no mundo todo, de modo a garantir com uma produção maior em uma área menor (FAO, 2016). Existe uma ampla diversidade de pesticidas com diferentes especificidades e que são usados em diferentes situações, os herbicidas à base de glifosato são atualmente os mais utilizados, tanto em nível nacional, quanto mundial (IBAMA, 2021; BENBROOK, 2016). A intensificação do uso do glifosato em atividades agrícolas, incluindo surfactantes, adjuvantes e seu principal metabólito, gera uma preocupação sobre o seu destino final nos ecossistemas aquáticos e contribui para o aumento desses compostos no ecossistema. De forma a investigar as possíveis rotas envolvidas com os efeitos do glifosato no ciclo de vida do peixe-zebra, foram realizados dois estudos: um estudo experimental controlado e uma revisão sistemática e meta-análise.

O primeiro estudo destaca pontos importantes para a literatura, pois avaliamos concentrações permitidas na água potável de um GBH na etapa embrionária e larval do peixe-zebra. Existe uma necessidade de se avaliar os efeitos de pesticidas em concentrações ambientalmente relevantes, uma vez que grande parte dos estudos de ecotoxicologia focam em avaliar concentrações comerciais ou acima delas. Sendo assim, constatamos que o GBH nas concentrações escolhidas (250 e 500 µg/L) causa prejuízo no desenvolvimento embrionário, como na mortalidade, malformações e eclosão. Na mortalidade observamos um aumento no número de embriões mortos nas primeiras 6 horas de exposição. Em relação às eclosões, houve um aumento em 48hpf nos grupos expostos ao glifosato. As malformações observadas como edema pericárdico, edema do saco vitelino, curvatura da coluna vertebral e malformações corporais (incluindo malformação da cauda, cauda curta e malformação da cabeça) receberam devida atenção especial. Pois, na literatura que apresenta essas malformações são encontradas em concentrações maiores de glifosato (100 µg/L, SULUKAN et al., 2017).

É valido mencionar que possuímos indícios fortes de que a formulação de glifosato nas concentrações utilizadas consegue passar pelos poros do córion e se acumularem no saco vitelino, devido ao aumento visualizado. Também, o sistema metabólico fica deficiente durante os primeiros dois dias nos embriões, podendo indicar essa acumulação (WU et al., 2017). Ainda, constatamos um aumento na frequência cardíaca nos animais expostos ao contaminante, esse aumento pode acarretar na morte de cardiomiócitos afetando o desenvolvimento do embrião (QIAN et al., 2018). Como descrito na literatura, o glifosato é amplamente conhecido por inibir enzimas, como GST e AChE (SANDOVAL-HERRERA et al., 2019). Mesmo em concentrações legalmente permitidas na água, observamos inibição dessas duas enzimas.

Os resultados do primeiro estudo destacam a ameaça a qual os organismos aquáticos estão suscetíveis, uma vez que eles podem ser expostos a uma ampla variedade de compostos tóxicos. Mesmo em concentrações legalmente permitidas na água potável, verificou-se alterações na morfologia, na mortalidade, eclosão, frequência cardíaca, atividade da AChE e GST. A adoção de valor limite máximo de resíduos pode dar à população uma falsa ideia de segurança (PETERSEN, 2015). Mas, como observado nos resultados apresentados nesse estudo, concentrações baixas do glifosato induziu alterações morfológicas, bioquímicas e no desenvolvimento dessa espécie.

O segundo estudo faz uma compilação de todas as pesquisas que abordaram os efeitos causados pelo glifosato em todas as etapas de vida do peixe-zebra. Observamos que na fase embrionária e larval ocorreu alterações na mortalidade, eclosão, malformações, morfologia e frequência cardíaca. Em relação a mortalidade houve um aumento em 3, 24, 48, 72 e 96 hpf. Nas taxas de eclosão, houve um aumento de 48 hpf e uma diminuição em 72 hpf. Constatamos que ocorreu um aumento nas malformações, que se trataram de edema do saco vitelino, edema pericárdico, curvatura da coluna e malformações corporais. Quanto à morfologia observamos diminuição do tamanho corporal. Em relação à frequência cardíaca, houve diminuição do número de batimentos em 48 e 72 hpf.

Já na fase adulta constatamos alterações bioquímicas e comportamentais. Os resultados bioquímicos demonstraram um aumento de ROS nas brânquias em 24 horas de exposição ao glifosato e aumento de ACAP nas brânquias em 96 horas de exposição. Além disso, na literatura já foi constatada alterações nas atividades enzimáticas no fígado como a catalase e glutationa peroxidase (SANTO et al., 2018; VELASQUES et al., 2016). Podendo então reduzir as defesas da célula e aumentar a sensibilidade ao glifosato. Também, ocorreram alterações nos níveis de tióis no cérebro e peroxidação lipídica no cérebro, fígado e músculos (LOPES et al., 2018; SANTO et al., 2018). Em relação ao comportamento, ocorreu uma diminuição da distância percorrida e a velocidade média dos animais e um aumento no número de rotações. Além disso, em outras pesquisas, foi constatado prejuízo na memória e redução no comportamento agressivo (BRIDI et al., 2017; DA COSTA CHAULET et al., 2019). Verificamos que houve relação entre o aumento da concentração de glifosato e a presença de distúrbios associados ao comportamento. Visto que, devido a ampla gama de aplicações desse herbicida em uma cultivar agrícola, as concentrações de glifosato no ambiente aquático podem estar em maiores concentrações nesses períodos (POHL et al., 2019).

7 CONCLUSÃO

Concluímos, a partir dos estudos apresentados que, há fortes evidências de que o glifosato e os herbicidas à base de glifosato são altamente tóxicos para o peixe-zebra. No estudo experimental, mostramos que o GBH em concentrações encontradas na água potável inibe a atividade de enzima chave na transmissão de impulso nervoso e na detoxificação em peixe-zebra na fase embrionária e larval. Além disso, observamos alterações no desenvolvimento, bem como, o aumento no número de malformações. Sendo esses efeitos, prejudiciais para as atividades básicas de sobrevivência e podendo afetar o animal na vida adulta. Nesse sentido, nossa revisão sistemática com meta-análise buscou evidenciar os efeitos do glifosato em todas as fases da vida do peixe-zebra. Demonstramos que o glifosato pode induzir na fase embrionária e larval aumento da mortalidade, malformações, afetar a eclosão e a frequência cardíaca. Já na fase adulta, foram observados danos bioquímicos e comportamentais. Sendo assim, estudos futuros que desvendem as vias afetadas pelo glifosato no peixe-zebra, acompanhando toda a fase da vida são urgentes. Além disso, torna-se importante avaliar os prejuízos causados na prole pela exposição parental do peixe-zebra ao glifosato. Por fim, nosso estudo reforça a atenção aos riscos da formulação comercial e pura do glifosato para a saúde dos peixes, destacando o uso de medidas de precaução e mitigação aos malefícios constatados.

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