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MINI-REVIEW

Effects of glyphosate on zebrafish: a systematic review and meta-analysis

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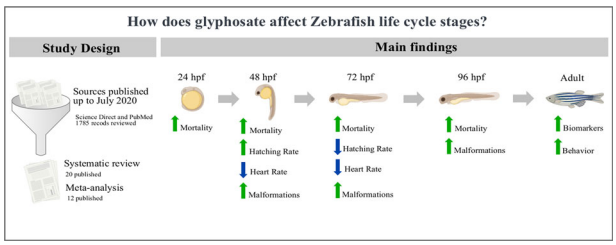
Abstract

Glyphosate herbicide is widely used in worldwide crop production. Consequently, its active ingredient, surfactants, and adjuvants commonly reach the aquatic ecosystem, thereby harming the biota. An investigation into how this herbicide affects aquatic species is important, especially in fish, as they have the ability to absorb and concentrate toxins. We aimed to evaluate the effects of glyphosate on the embryonic, larval and adult stages of zebrafish (*Danio rerio*), an appreciable organismal model. In this sense, we performed a meta-analysis using published articles from online databases (PubMed and ScienceDirect), which covered studies published until 2022. From a massive compilation of studies evaluating the effects of active substance glyphosate and Glyphosate-Based Herbicides (GBH) on zebrafish, we selected 36 studies used in downstream analyses. Overall, we report that glyphosate affects developmental stages and demonstrates toxicity and damage in zebrafish. We observed that embryos exposed to glyphosate exhibit increased mortality. There was also an increase in the number of morphological abnormalities related to yolk sac oedema, pericardial oedema, spinal curvature and body malformations, and a decrease in body size was observed. Furthermore, there was a decrease in the number of beats. The biochemical results demonstrated an increase in reactive oxygen species and antioxidant capacity against peroxyl radicals in the gills. The literature shows that glyphosate decreased the distance covered and the mean speed of the animals and increased the number of rotations. We concluded that glyphosate causes damage in the embryonic, larval and adult stages of this species. These results are valid for zebrafish and can be applied to other freshwater fish species.

Graphical abstract



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Introduction

The demand and production of pesticides to control unwanted organisms has increased annually worldwide (Zhang and Liu 2017), mainly motivated by the advances in agricultural technology and the demand for greater productivity. Although pesticides may benefit agricultural production, a plethora of ecosystem damage is routinely recorded in empirical 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 (that is, herbicide) is glyphosate [Glyphosate-Based Herbicides (GBH)]. Since its first commercialisation in the seventies (Baird 1971), GBH has been the most widely used herbicide in the world (Benbrook 2016). For example, until 2014, around 800 million kilograms of GBH were used worldwide, with estimates expected to reach 740 to 920 thousand tons by 2025 (Benbrook 2016; Maggi et al. 2020). In the context of agriculture, glyphosate is mixed with chemical ingredients, such as adjuvants and surfactants (for example, polyoxyethylene amine – POEA and alkyl polyphosphate amine) (FAO 2016). In turn, when disposed in the natural environment, glyphosate can be decomposed into two metabolite types, namely aminomethylphosphonic acid (AMPA) or sarcosine (Borggaard and Gimsing 2008).

The intensified use of glyphosate in agricultural activities, including surfactants, adjuvants and its main metabolite, increases the presence 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 and 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). This is concerning, as 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 case of fishes, the glyphosate can be absorbed by gills and through 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 acetylcholinesterase (AChE) activity (Glusczak et al. 2011, 2007; Lushchak et al. 2009). In this sense, the use of zebrafish represents an adequate alternative to study the effects of pesticides on water. Zebrafish is an ideal bioindicator of water quality, as it exhibits a rapid development, has a size that is suitable for maintenance (3 to 4 centimetres) and high fertility and meets the 3 Rs criterion (Grunwald and Eisen 2002). Another feature intrinsically associated with zebrafish is that different stages of its development may be evaluated in toxicological studies (embryo: 0–48 h; 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 favourable for use in ecotoxicological studies.

Although specific studies have revealed the 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 absent. Therefore, a framework that integrates sophisticated analytical approaches to systematically assessing the effects of glyphosate can provide an overview of zebrafish's sensitivity to the herbicide, with implications relevant to freshwater ecosystems as a whole. Here we performed a systematic review followed by a meta-analysis of glyphosate toxicity during 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.

Methods

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 ScienceDirect full text search, to identify studies that 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 May 2022, and the keywords were searched in the English language. This yielded a total of 3394 results (see Results section for more details). We initially screened studies based on titles and abstracts. Thereafter, we read the full text to extract all the necessary information.

Some studies were not included in the meta-analysis, only in the review, as they presented insufficient data for comparison. In addition, those that did not provide adequate information regarding the type of pesticide used and/or studies with restricted access to the full text were excluded. Reviews, PhD theses, scientific notes and book chapters were not included. We also disregarded articles in which the active ingredients of different pesticides were combined. In this sense, the articles included met the following criteria: (1) original research, performed with zebrafish exposed to glyphosate; (2) glyphosate 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) or mean and standard error (MD ± SE).

To summarise, 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 grouped the obtained data into effects caused by glyphosate on 1) mortality (for example, in hours post fertilisation – hpf), 2) hatched (for example, in hpf), 3) malformation (for example, yolk sac oedema), 4) morphology (for example, body length), 5) heart rate (for example, in hpf), 6) biochemistry (for example, ACAP) and 7) behaviour (for example, rotations).

Data extraction

All search results considered were tabulated in a digital spreadsheet (using Microsoft Excel). The following information was included in this table: authors, year of publication, country, stage of life, active substance glyphosate or GBH, concentration, replacement, dilution, the effect caused, specification of effect and temperature. All extracted information was standardised to the same unit of measure (for example, heart rate per minute). Studies, which tested different concentrations of glyphosate for the same control, were considered as independent sampling units. Although different glyphosate formulations are known, we consider the combined effect of different GBHs and active substance glyphosate, as the data are insufficient to perform separate meta-analyses. However, the active substance glyphosate and its adjuvants can be found together in the aquatic environment. Therefore, we treat the results keeping the combination in mind throughout the manuscript – an approach that has been followed in previous studies (see Battisti et al. 2021 for similar approaches).

We also extracted the sample size (n), mean and standard deviation for the control and experimental groups for each article. When the results were presented in terms of standard error of the mean (SEM, standard error of the mean), we converted this into standard deviation (SD) using an equation that has been previously described in literature (Vesterinen et al. 2014). We extracted mean and standard deviation from a procedure performed using the FIJI software ImageJ (Schindelin et al. 2012).

Data analysis

We performed a meta-analysis on the sample size, mean and standard deviation in order to calculate a standardised measure for each study (that is, effect size). Subsequently, we used the R package meta (Balduzzi et al. 2019) to calculate the standardised mean difference (SMD) as an estimate of effect size on two types of outcomes – continuous and binary data. We regard those instances in which studies have evaluated the effect of glyphosate on quantitatively measured structures as continuous outcomes. In contrast, we consider the instances that have been categorically measured as binary outcomes.

For continuous outcomes (adult: biochemistry and behaviour; 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). The SMD (effect size) was considered significant when the 95 per cent confidence interval (CI) did not include zero. We also quantified and tested for statistical heterogeneity using Higgin's I^2 (Higgins et al. 2003). Moreover, we considered a sub-group analysis to determine the effects of glyphosate based on the hours post fertilisation (hpf) or some specification parameter dependent (see forest plot legends for more details on the sub-group used). Calculated effect sizes and 95 per cent CI were used to generate forest plots. The results were considered significant at the level of $p < 0.05$. 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 occurrence of malformations), we calculated the fixed and random effect 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 the Odds Ratio (OR) with a 95 per cent CI (Mantel and Haenszel 1959; Egger et al. 2001). The results were considered significant at the level of $p < 0.05$. We also evaluated the degree of residual heterogeneity in our data using Higgin's I^2 statistics (Higgins et al., 2003). Similarly, for 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 created meta-regression models using the "metareg" function. We also checked the publication bias in our dataset from three approaches. First, we analysed the 10-item checklist of the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE), which is 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 entails using a quantitative method to test for asymmetry in the funnel plot (Egger et al. 1997). All aforementioned functions were derived from the 'meta' package (Balduzzi et al. 2019) in R version 4.0.3.

Results

Systematic search

Database searches resulted in an initial total of 3394 documents ($n = 247$ from PubMed, and $n = 3147$ from ScienceDirect) through systematic search (Fig. S1). Duplicates ($n = 2024$) and other research subtypes such as reviews and book chapters ($n = 885$) were removed. Furthermore, studies that analysed the effects on other living beings ($n = 334$) and studies with other contaminants with the exception of glyphosate ($n = 68$) were rejected. After these exclusions, we obtained a total of 83 articles, which were subjected to a full text reading. In addition, in vitro studies ($n = 35$) evaluated the effects of exposure of zebrafish cells to glyphosate, effects of other variables with glyphosate, such as pH and sediment ($n = 11$) and without statistical analysis ($n = 1$). We could not gain access to the full text of one of the articles, despite searching in all access routes. After selection by title, abstract and full text, 36 original research articles with zebrafish exposed to glyphosate commercial and/or pure were obtained. All these studies were included in the systematic review, and the main characteristics and results are presented in Table S1.

Study characteristics

Taken together, the studies included in this review were published between 2014 and 2022. These studies were carried out in Brazil ($n = 14$), China ($n = 4$), India ($n = 1$), Portugal ($n = 3$), Turkey ($n = 1$), United Kingdom ($n = 1$), Czech Republic ($n = 1$), United States ($n = 5$), Mexico ($n = 2$), France ($n = 1$), Ukraine ($n = 1$), Canada ($n = 1$) and Spain ($n = 1$). The articles are detailed in Table S1. Of these, 21 articles used zebrafish in the embryonic and larval stages, and 15 studies used zebrafish in the adult stage, two of which evaluated the embryonic, larval and adult stages. The dosages of GBH and active substance used ranged from 0.00005 to 400 mg/L. The active substance used was approximately 99 per cent pure and the GBHs were quite diverse. All selected studies show that exposure of zebrafish, regardless of its stage of life, to some GBHs or active substance induces damage to this fish species.

The findings showed that glyphosate affects development in the embryonic and larval stages. The studies evaluated the mortality and hatching rate of embryos and larvae through exposure to GBHs and active substance at concentrations ranging from 0.00005 to 400 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; Zhang et al. 2017; Forner-Piquer et al. 2021; Díaz-Martín et al. 2021; Díaz-Martín et al. 2021; Pompermaier et al. 2022). The heart rate was also verified to check cardiovascular damage (Gaur and Bhargava 2019; Lanzarin et al. 2019; Roy et al. 2016b; Zhang et al. 2021; Liu et al. 2022; Pompermaier et al. 2022; Díaz-Martín et al. 2021; Díaz-Martín et al. 2021). Changes in morphology and malformations were evaluated through exposure to GBHs and active substance ranging from 0.00005 to 35 mg/L (de Brito Rodrigues et al. 2019; Roy et al. 2016a, 2016b; Sulukan et al. 2017; Zhang et al. 2017; Forner-Piquer et al. 2021; Liu et al. 2022). Biochemical damage was discovered 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; Lanzarin et al. 2021; Liu et al. 2022; Pompermaier et al. 2022). Several researchers also verified the effects on behaviour through changes in locomotor activity, aversive behaviour, rotation and other analyses (Bridi et al. 2017; Zhang et al. 2017; Forner-Piquer et al. 2021; Ivantsova et al. 2022). In addition, they found an increase in the gene expression of pax2a, kim1, sod2, cox4i1 and so on (Babich et al. 2020; Ivantsova et al. 2022; Zhang et al. 2021).

Regarding the effects of glyphosate on adult zebrafish, mortality, survival, biochemical and behavioural changes were verified. Two studies evaluated the mortality and survival of animals throughout the experiment (Davico et al. 2021; Falfushynska et al. 2022). During the biochemical analysis, the authors found damages in the gills, liver, brain and muscle in the form of biomarkers such as SOD, CAT, GPx, ROS, ACAP and 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; Faria et al. 2021; Ding et al. 2021; Falfushynska et al. 2022; Giommi et al. 2022). Furthermore, the authors noted effects on behaviour, such as distance travelled, latency to enter the upper zone, average speed, rotations, distance covered, avoidance and so on (Bridi et al. 2017; da Costa Chaulet et al. 2019; da Rosa et al. 2016;

Pompermaier et al. 2020; Faria et al. 2021; Mena et al. 2022).

Study quality and risk of bias

Supplementary Fig. S2 presents the 'high' or 'low' risk of bias in terms of percentages for each domain assessed for risk of bias within each individual study and across studies, respectively. In 100 per cent of the studies ($n = 36$), 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, thereby demonstrating a homogeneity of animals between groups. As for allocation secrecy, 100 per cent of the studies ($n = 36$) did not report whether the group allocation was adequately concealed, and 80,5 per cent of the studies ($n = 29$) did not mention the random housing of the animals. The random allocation of animals is not yet a standard practice in animal experiments and it may reflect a possible distortion of the global interpretation of the data. In all studies (100 per cent, $n = 36$), there were no reports about the lack of knowledge regarding the interventions utilised by caregivers. In addition, there were no reports regarding 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 per cent, $n = 36$) did not present other potential sources of bias and incomplete results. In this sense, the objects of comparison (variables) were potentially approximated and analysed with greater certainty. Finally, 91,6 per cent of the studies ($n = 33$) had a conclusion free of selectivity. Thus, we started with the statistical procedures described, to build a body of evidence on the problem in question.

Meta-analysis

Glyphosate affects mortality and hatching in zebrafish embryos

We observed that embryos exposed to glyphosate exhibit an increase in mortality in 3 hpf ($I^2 = 63$ per cent; $p < 0.01$), 24 hpf ($I^2 = 91$ per cent; $p < 0.01$), 48 hpf ($I^2 = 93$ per cent; $p < 0.01$), 72 hpf ($I^2 = 95$ per cent; $p < 0.01$) and 96 hpf ($I^2 = 75$ per cent; $p < 0.01$) (Fig. 1). Thus, the number of dead animals during the entire embryonic and larval stages is higher in groups exposed to this pesticide. The visual inspection of the funnel plot in Supplementary Fig. 3A and the Egger test ($t = 2.139$, $p = 0.0374$) indicates the possible presence of publication bias in our results. We performed a meta-regression to check whether there was a relationship between mortality and the dosage of glyphosate or GBH used. The target regression demonstrated a significant positive relationship between embryo mortality and the dosage

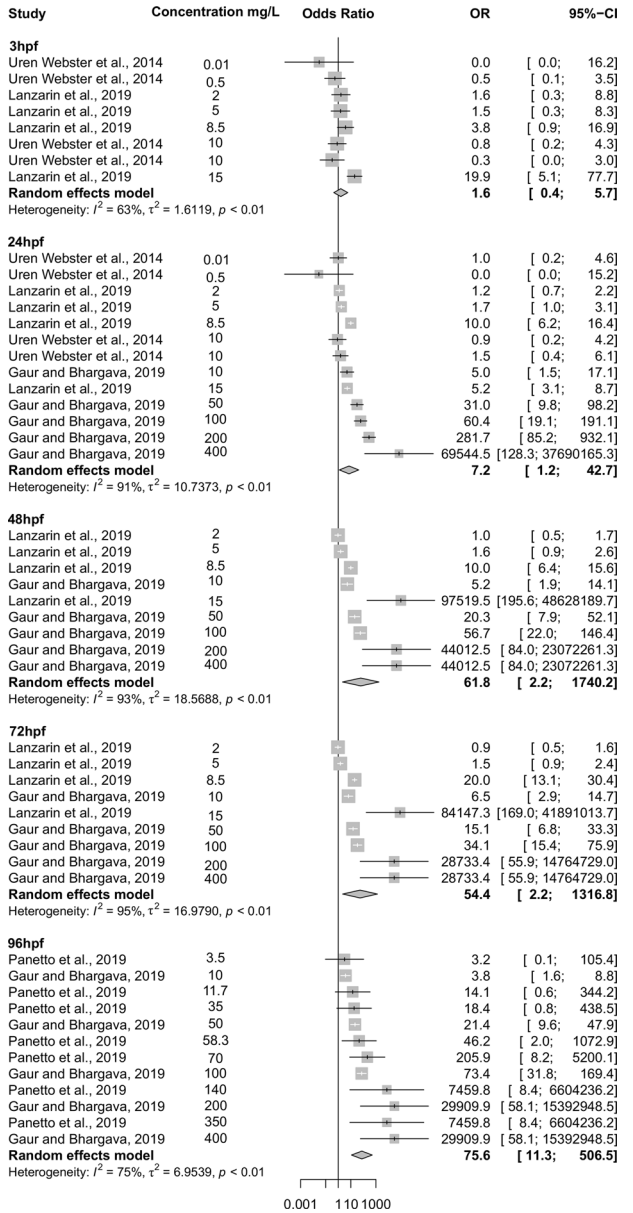

Fig. 1 Forest plot of odds ratios (OR) and 95% confidence intervals (CI) for the effect of glyphosate concentration on mortality in zebrafish embryos and larvae. We considered a sub-group analysis based on the hours post fertilisation

used (se = 0.0031, $p < 0.0001$, Supplementary Fig. 4A). The higher the concentration of glyphosate used, the greater is the probability of observing an effect on mortality.

We found mixed results regarding the effects of glyphosate on hatching rates, whereby there was an increase in 48 hpf ($I^2 = 72$ per cent; $p < 0.01$) and a decrease in 72 hpf ($I^2 = 94$ per cent; $p < 0.01$), and there was no difference in 96 hpf ($I^2 = 0$ per cent; $p = 1.00$) (Fig. 2). We observed that glyphosate accelerates the hatching of embryos at 48 hpf. A visual inspection of the funnel plot (Supplementary Fig. 3B) and the Egger test ($t = 1.907$, $p = 0.027$) indicates no evidence of publication bias in our results. We observed that

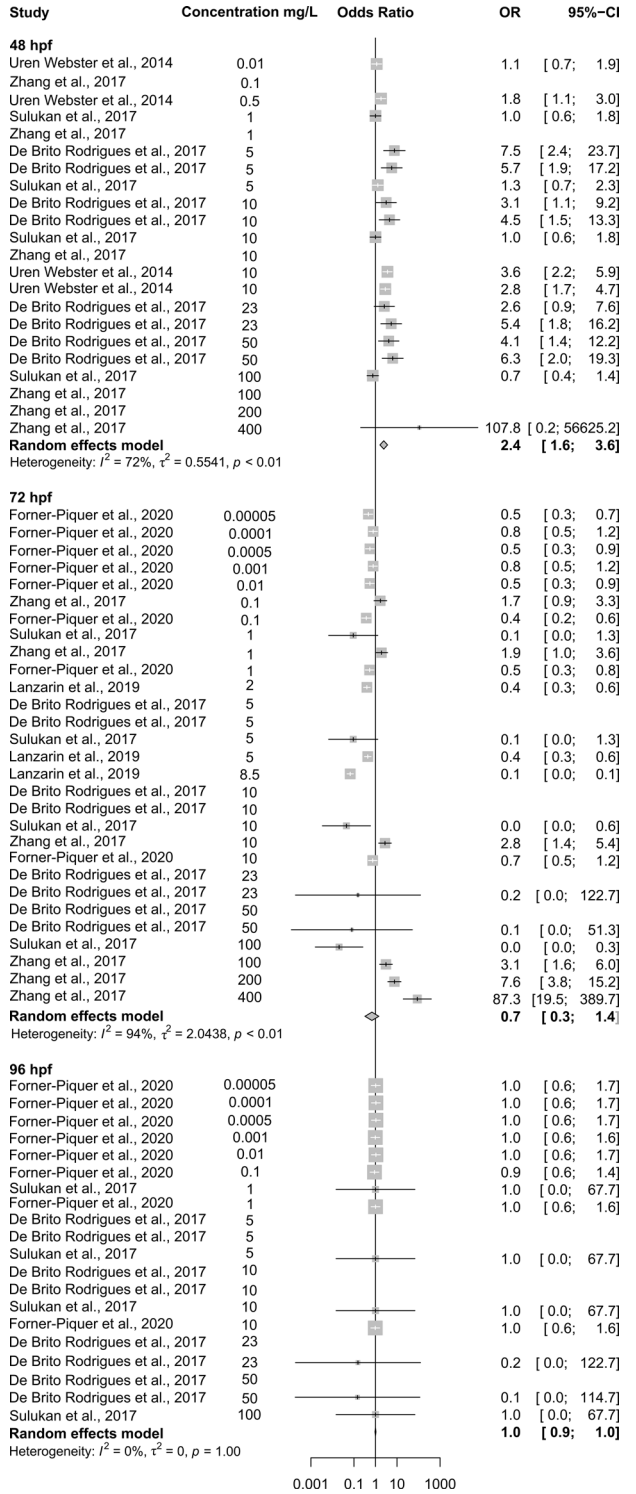

Fig. 2 Forest plot of odds ratios (OR) and 95% confidence intervals (CI) for the effect of glyphosate concentration on hatching in embryo and larva zebrafish. We considered a sub-group analysis based on the hours post fertilisation

the meta-regression showed a significant positive relationship between the hatch rate and the dosage of GBH or active substance used (se = 0.8630, $p < 0.0001$, Supplementary Fig. 4B).

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 oedema ($I^2 = 80$ per cent; $p < 0.01$), pericardial oedema ($I^2 = 75$ per cent; $p < 0.01$), spinal curvature ($I^2 = 77$ per cent; $p < 0.01$) and body malformations ($I^2 = 88$ per cent; $p < 0.01$) (Fig. 3). A visual inspection of the funnel plot (Supplementary Fig. 3C) and the Egger test ($t = 1.34$; $p = 0.1919$) indicates no evidence of publication bias in our results. It was not possible to analyse this parameter by meta-regression, owing to insufficient observations.

In relation to morphology, we regarded changes mainly associated with a decrease in body size in animals exposed to glyphosate ($I^2 = 98$ per cent; $p < 0.01$, Fig. 4). A visual inspection of the funnel plot (Supplementary Fig. 3D) and the Egger test ($t = 7.051$, $p = 0.0000014$) indicates the possible presence of publication bias in our results. Meta-regression demonstrated a significant positive relationship in glyphosate-exposed embryos on morphology (se = 0.4946, $p < 0.0001$, Supplementary Fig. 4C).

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 ($I^2 = 99$ per cent; $p = 0$) and 72 hpf ($I^2 = 100$ per cent; $p = 0$) (Fig. 5). A visual inspection of the funnel plot (Supplementary Fig. 3E) and the Egger test ($t = 2.89$, $p = 0.008$) indicates the possible presence of publication bias in our results. Meta-regression demonstrated a significant negative effect on heart rate in the case of glyphosate-exposed embryos (se = 4.4679, $p < 0.001$, Supplementary Fig. 4D). We found that, at lower concentrations, there is a greater likelihood that we will perceive an effect on the heart rate.

Glyphosate changes the reactive oxygen species (ROS) and antioxidant capacity against peroxyl radicals (ACAP) 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 ($I^2 = 88$ per cent; $p < 0.01$; Fig. 6). In addition, it increased the antioxidant capacity against peroxyl radicals (ACAP) in the gills within 96 h of exposure ($I^2 = 61$ per cent; $p = 0.05$). A visual inspection of the funnel plot (Supplementary Fig. 3F) and the Egger test ($t = -2.359$, $p = 0.0333$) indicates the possible presence of publication bias in our results. It was not possible to analyse this parameter by meta-regression, owing to insufficient observations.

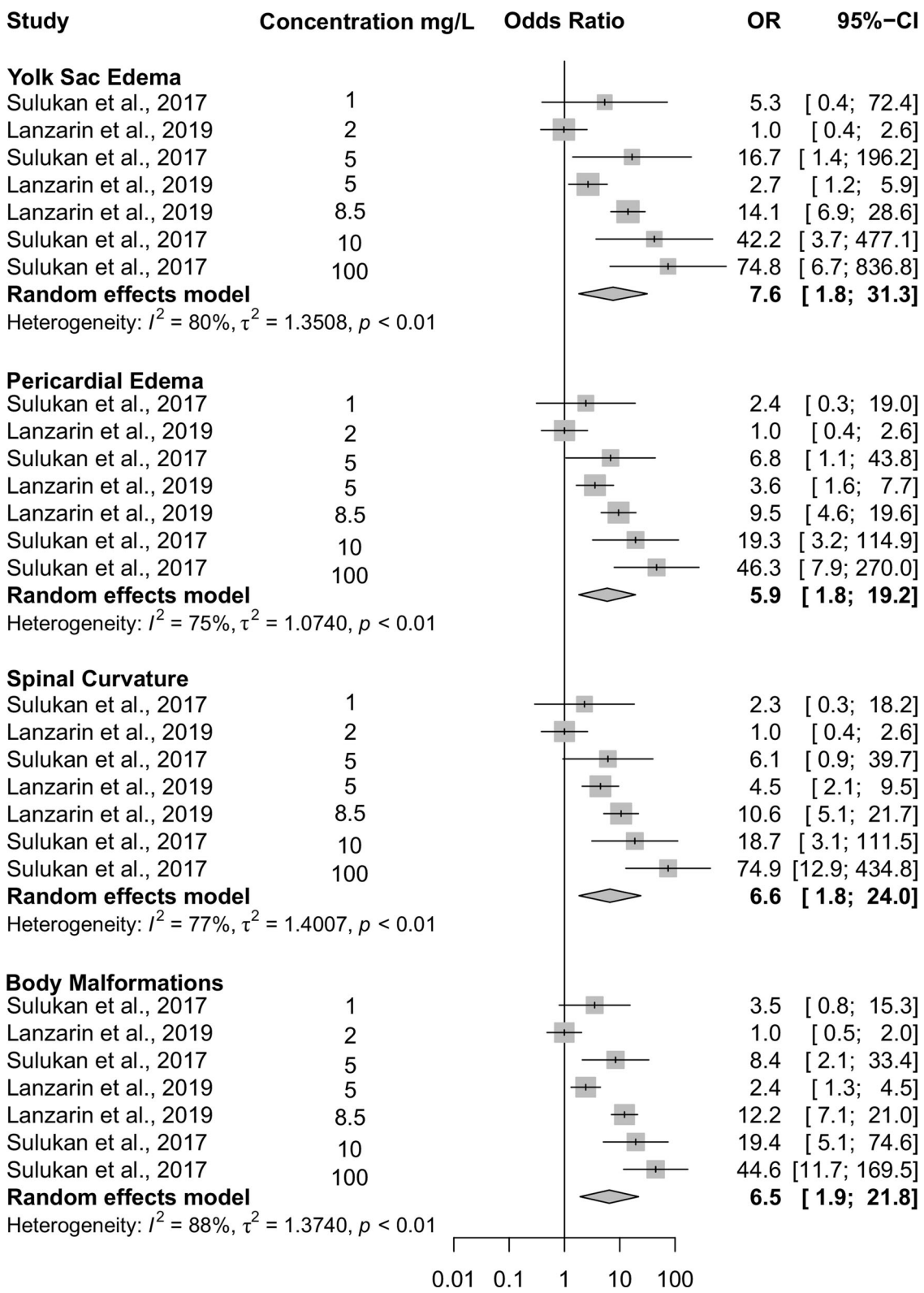

Fig. 3 Forest plot of odds ratios (OR) and 95% confidence intervals (CI) for the effect of glyphosate concentration on malformation in embryo and larva zebrafish. We considered a sub-group analysis based on the structure of body

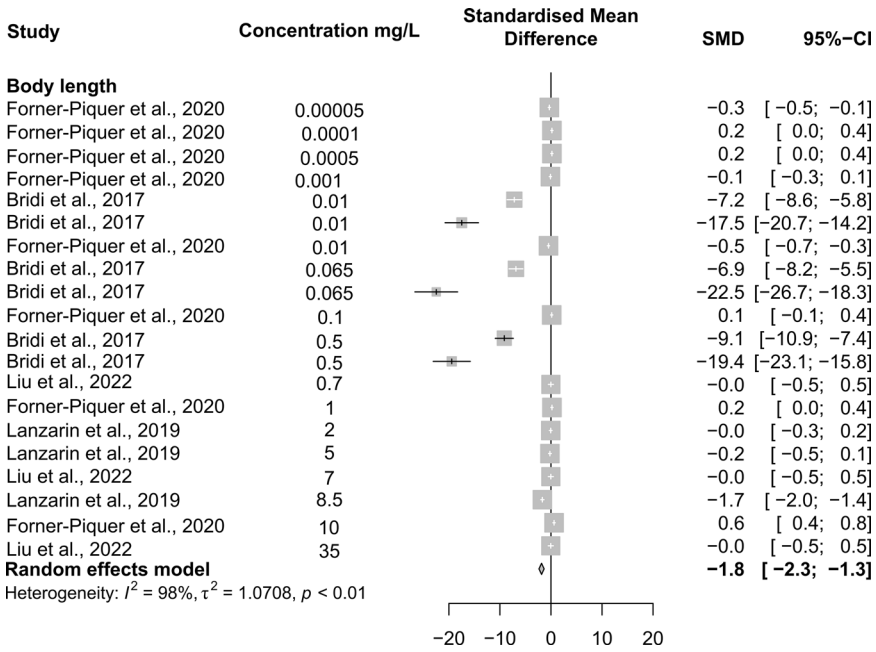

Fig. 4 Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CI) for the effect of glyphosate concentration on morphology in embryo and larva zebrafish. We considered a sub-group analysis based on the structure of body

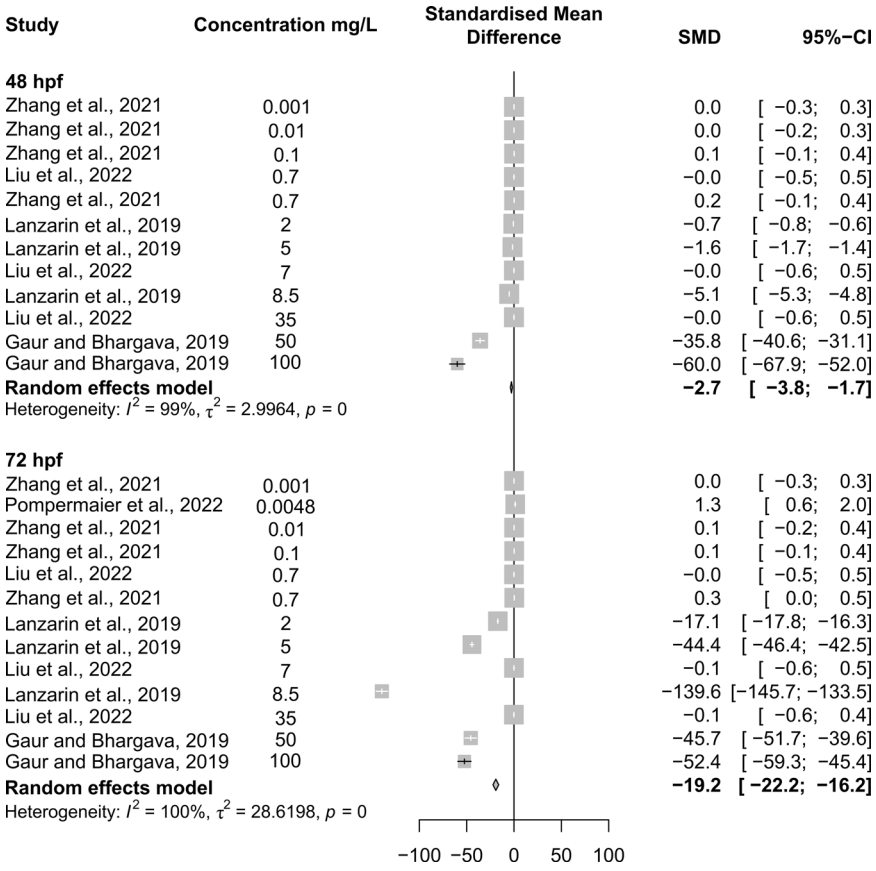

Fig. 5 Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CI) for the effect of glyphosate concentration on heart rate in embryo and larva zebrafish. We considered a sub-group analysis based on the hours post fertilisation

Glyphosate alters the behaviour of adult zebrafish

We found that glyphosate decreased the distance travelled ($I^2 = 99$ per cent; $p < 0.01$) and the average speed of the animals ($I^2 = 88$ per cent; $p < 0.01$). In addition, exposure to glyphosate increased the number of rotations ($I^2 = 98$ per cent; $p < 0.01$) (Fig. 7). A visual inspection of the funnel plot (Supplementary Fig. 3G) and the Egger test ($t = 2.368$, $p = 0.0280$) indicates the possible presence of publication bias in our results. The meta-regression demonstrated a

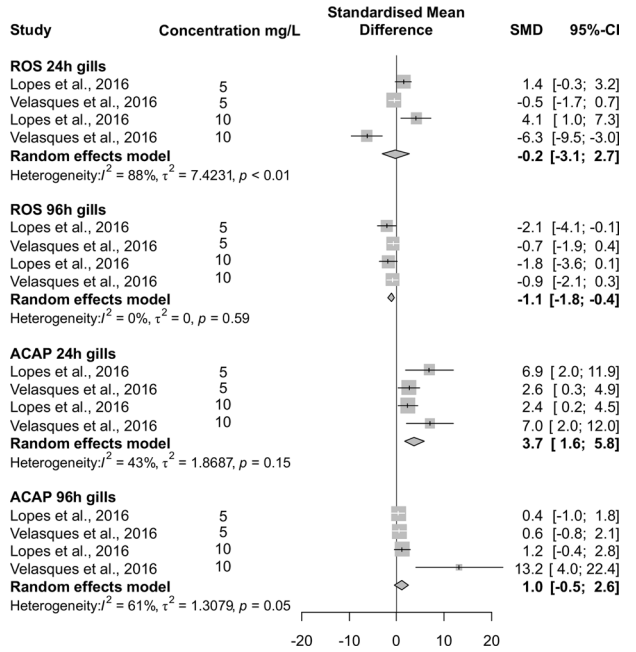

Fig. 6 Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CI) for the effect of glyphosate concentration on biochemistry in adult zebrafish. We considered a sub-group analysis based on the biomarkers

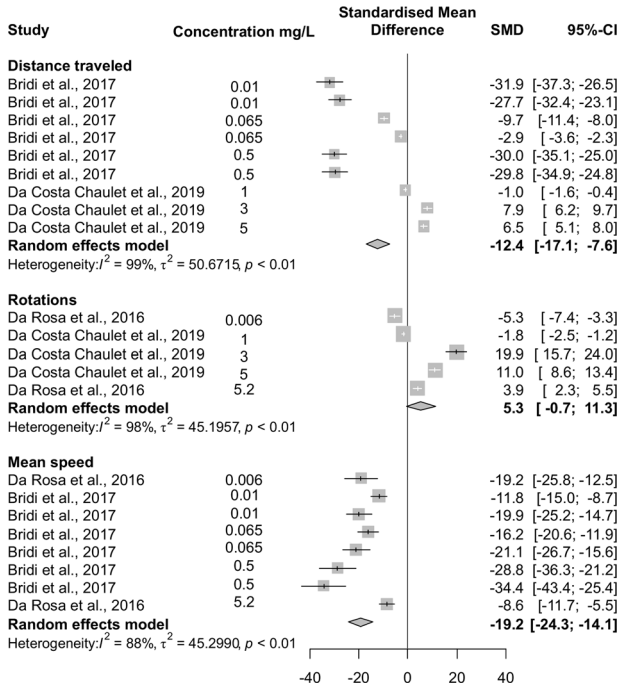

Fig. 7 Forest plot is indicating the mean difference (SMD) and 95% confidence intervals (CI) for the effect of glyphosate concentration on behaviour in adult zebrafish. We considered a sub-group analysis based on the type of behaviour

significant positive relationship between adult behaviour and the concentration used (se = 0.6598, $p < 0.0001$, Supplementary Fig. 4E). The higher the concentration of glyphosate used, the greater is the probability of observing an effect on behaviour.

Discussion

The damage caused by the toxic effects of glyphosate and its additives has been widely reported in the *corpus* of scientific literature. The use of zebrafish (*Danio rerio*) as a model for the mechanistic description of its action as well as the ecological implications of using this pesticide have considerably increased in the recent years. Thus, we chose to use the results of works published until 2022 in reference to this species in vivo, thereby compiling the stages of its life cycle (embryonic, larval and adult). Studies show 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), thereby demonstrating the need for a broader look at these effects. However, there was no record of experimental trials that considered the damage caused by glyphosate during all stages of the lifecycle of the zebrafish fish species. In the natural environment, the fish species comes into contact with the pesticide during 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). In the United States, 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. It should be mentioned that the dosages of GBH and active substance used in the studies included in this review ranged from 0.00005 to 400 mg/L. In addition, most studies used concentrations that are not environmentally relevant. Therefore, in the present study, we included all publications, which pertained to the exposure of zebrafish to GBH and/or active substance, in a single document. It is important to mention that there are a lot of GBH formulations around the world. Furthermore, it is very difficult to attribute the toxic effects to the active substance per se due to variations in the commercial formulations available. These GBH formulations include ammonium salt, glycine salt and other chemical substances used to improve plant absorption of the herbicide (Benbrook 2016).

Exposure of fish embryos to GBH presents an increased danger, as 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 0.5–0.7 µm thick with three layers perforated by pore channels in fertilised eggs (Bonsignorio et al. 1996; Rawson et al. 2000). It acts as a barrier to protect embryos from external stimuli (Tran et al. 2021) and 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 active substance 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, during which cell division occurs (Kimmel et al. 1995). This is the reason behind why this period becomes the most critical for the survival of the embryo itself. The high sensitivity of the organism to glyphosate toxicity during this period was evident. In our meta-analysis, we observed 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 GBH (Lanzarin et al. 2019). It is important to highlight that there are more environmental interferences, in addition to glyphosate, in the natural environment. However, the increased 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. Furthermore, there was a pronounced increase in mortality in embryos with 22 hpf, which were exposed to 8.5 and 15 mg/L of GBH (Lanzarin et al. 2019). This high mortality is related to developmental delay and embryo malformations. In our work, it was evident that embryos exposed to glyphosate have a smaller body size. This evidence has been reinforced in a study that evaluated gene expression *ntl (no tail)*, which is responsible for the formation of the notochord, which demonstrated that glyphosate could 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 is the likelihood of anatomical changes, such as body length.

With reference to 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 is the probability of observing these anatomical damages. Interestingly, these malformations, induced by glyphosate, include oedema 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 analysed, morphological abnormalities are frequently recorded in a wide spectrum of active substance 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).

The reason behind the formation of oedema 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). Alternately, these oedemas 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 oedema to interference in the synthesis and metabolism of lipids present in the yolk sac. Owing 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. Consequently, malformations in the body were observed. With reference to the damage to the formation of the spine, we can list the following: i) an over-expression of growth hormone; 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); 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. These effects may cause changes in locomotor activity and aversive behaviour after exposure to active substance or Roundup® (GBH), 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 faced while searching for food and finding partners for reproduction. These factors promote impaired development, which, in turn, 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. In addition, we discovered that mortality 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 GBH (Lanzarin et al. 2019).

With respect to 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 0.01 mg/L and 400 mg/L. Our findings allowed us to observe that the higher the concentration of glyphosate used, the greater is 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 GBHs, 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. The reported period for the normal hatching of developing embryos is between 48 and 72 hpf (Kimmel et al. 1995). Therefore, even if the hatch occurred in a time interval considered normal, we found that there was an increase in the number of hatches in the exposed animals, thereby highlighting the interference of the contaminant. While hatching occurs due to the activity of proteolytic enzymes in specialised cells, hormonal metabolic patterns, and the movements of muscle contractions performed by the embryo (Samaee et al. 2015), the increase in hatching rates during this period may probably be attributed to alterations in proteolytic enzymes. Among the candidates, the incubation 1 enzyme (HE1) can be pointed out as the enzyme responsible for breaking the chorionic barrier (Sano et al. 2008).

With reference to the decrease in heart rate of 48 hpf, we showed that it occurred in animals exposed to concentrations between 2 mg/L and 100 mg/L of glyphosate. Different studies found a decrease in the 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*), thereby altering calcium homoeostasis through the expression of *Heat Shock Protein Family B (Small) Member 11* (hspb11) involved in the signalling pathway of nitric oxide (NO) (Gaur and Bhargava 2019). In addition, another reason behind this phenomenon may be the presence of acetylcholinesterase (AChE) inhibitors in the GBHs, which increase the concentration of acetylcholine in the synaptic cleft, cause continuous acetylcholine receptor stimulation and decrease the heart rate (Lin et al. 2007).

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). In association with these results, we found an increase in mortality in animals exposed to glyphosate at 72 hpf. There is evidence that shows an increase in mortality in embryos with 72 hpf at concentrations of 8.5 mg/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 the ventricle with the rupture of the cardiac wall, thereby leading to the 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. Thus, due to the oedema found in the yolk sac, the normal functioning of the heart is strongly affected, thereby leading to a decrease in heart rate through the reduction of 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, thereby affecting energy production, which is fundamental to the development of the embryo. We also have to consider that oedema was not always observed in all the studies included in this review. Therefore, we must be a little careful when making the blanket statement that this damage has occurred in all embryos.

There is an explicit 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 that is used to assess the interference of chemicals in embryo development (OCDE 2013). Therefore, embryos can become more vulnerable to predatory attacks, mechanical and osmotic stress and chemical compounds present in the external environment, which, in turn, render 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, during this same period, the larvae had lower body length than expected and the head and eyes reduced in size (Zhang et al. 2017). The *carbonic anhydrase* activity (EC 4.2.1.1) and *hexokinase* (EC 2.7.1.1) decreased, and an increase in apoptotic response was observed (Panetto et al. 2019; Sulukan et al. 2017).

Unlike larvae, in adult specimens, it was possible to analyse the specific effects of the organ from the perspective of the methodological peculiarity of the assessment of biochemical responses. Animals exposed to glyphosate showed an increase in ROS in the gills (exposed for 24 h),

and an increase in antioxidant capacity against *peroxyl* radicals in the gills (exposed for 96 h) was observed. Adult fish show changes in brain thiol levels as well as increased lipid peroxidation markers in the brain, liver and muscle (Lopes et al. 2018; Santo et al. 2018). The activities of enzymes *catalase* (EC 1.11.1.6) and *glutathione peroxidase* (EC 1.11.1.9) showed affected activities in the liver, increase in Antioxidant Capacity Against Peroxyl radicals (ACAP) and Reactive Oxygen Species (ROS) in the gills and the liver (Santo et al. 2018; Velasques et al. 2016). Thus, these enzymatic alterations reduce cellular defences, thereby increasing the susceptibility to toxic xenobiotic substances that would be metabolised under normal conditions as well as impairing gas exchange and osmoregulation.

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

The significant changes found in this review, which have been 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 being said, the data show that, owing to the impact on the embryonic stage, the chances of mortality increase, or else, complications will continue to persist throughout the life span of the fish species. The resulting impacts can compromise the search for food, the local reproduction and perpetuation of the species and the socialisation.

Although limitations are inherent in systematic review and/or meta-analysis studies, some caveats underlying our analyses need to be considered. First, our compiled dataset covered a small number of studies, which may have made some comparisons difficult. Notably, we recognise that the effects of glyphosate on zebrafish may differ between GBH and active substance. Another caveat in some cases is uncertainty regarding whether the reported effects can only be attributed to the specific impact of the active substance or the adjuvants present in GBH. Furthermore, care should be taken when generalising effects observed at the intracellular level by making extrapolations at the population level. However, owing to the low number of comparable studies in the final sample, it was not possible to separate the effects of glyphosate in the GBH or active substance. Keeping these caveats in mind, we conducted these analyses using methods that seek to minimise the risk of bias in the results and conclusions.

Conclusion

We concluded that GBH and active substance glyphosate can be considered toxic for the development of *Danio rerio*. In the embryonic and larval stages, they induce an increase in mortality, cause malformations in the body and affect hatching and heart rate. At high concentrations, the revised studies show a greater likelihood of observing an effect on morphology, hatching, malformations and mortality. In addition, the revised studies show this biochemical and behavioural damage, which is caused in the adult stage of this fish's lifecycle.

We believe that the revised studies indicate damage mainly caused 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 may undergo decline in populations. Therefore, precautionary and damage mitigation measures must be taken in a timely manner to reduce the potential risks of glyphosate to fish and other aquatic species. Hence, our study reinforces the necessary and urgent attention to the risks of glyphosate and its commercial formulations for the health of fish and the entire aquatic system.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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