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RESEARCH ARTICLE

Impact of glyphosate herbicide exposure on sperm motility, fertilization, and embryo-larval survival of pejerrey fish (*Odontesthes bonariensis*)

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Abstract
The herbicide glyphosate is widely used in agricultural practices around the world, can reach aquatic environments, and potentially impact non-target organisms. This study aimed to investigate the effects of glyphosate exposure (both as the active ingredient and its formulated product) on sperm quality, fertilization success, and development of pejerrey (*Odontesthes bonariensis*), a native freshwater fish species from Argentina. Results revealed a statistically significant increase in sperm motility at the highest concentration of the formulated product. In contrast, exposure to the active ingredient resulted in a decrease in certain motility parameters. Fertilization assays and embryonic development showed no notable effects in exposed groups. There were no effects in the morphology or temporal evolution of the embryonic stages, nor in the hatching rate. In contrast, larvae exposed to the formulated product exhibited a significant increase in mortality rates, reaching 100% mortality at the highest concentration within a few hours. These findings suggest differential susceptibility between embryos and larvae to glyphosate exposure and highlight the importance of simultaneously assessing the impacts of both the active ingredient and the entire formulation of glyphosate on freshwater fish reproduction and development.

Keywords Glyphosate · Fertilization · Sperm quality · Motility · Embryo and larval survival · Freshwater fish · Pesticides

Introduction

Agrochemical pollution is one of the most serious concerns for the conservation of aquatic ecosystems. The expansion of agricultural frontiers, the adoption of intensive agricultural practices, and the utilization of technological packages that include genetically modified crops have led to a parallel increase in pesticide use (Aparicio et al. 2017). In Argentina, the soybean cropped area increased from approximately 6 million ha in 1994/95 (before the introduction of GM glyphosate-tolerant crops) to 16 million ha in 2021/22, and the corn cropped area from 3 million to over 10 million ha in the same period (MAGyP 2023). The increase in pesticide use (including herbicides plus insecticides, fungicides, and acaricides, among others) was from 38 thousand tons to more than 240 thousand tons of active ingredients (a.i.), while the application per unit of cropland increased from 1 to more than 7 kg/ha between 1995 and 2020 (FAO 2023).

Glyphosate (N-(phosphonomethyl) glycine (PMG)), a broad-spectrum herbicide, has emerged as one of the most widely used pesticides in this region for controlling a wide range of weeds in soybean and corn crops. However, the extensive use of glyphosate has raised concerns about its potential environmental impacts, particularly on non-target species and aquatic ecosystems.

The physicochemical properties of PMG, such as its high solubility in water and strong binding capacity to soil organic matter, explain its rapid and widespread distribution, as well as its persistence and retention in aquatic

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environments. Factors such as wind, rain, or irrigation that increase infiltration and surface run-off can facilitate this herbicide mobilization. Moreover, countries with extensive genetically modified crop areas have reported high application rates of glyphosate-based herbicides, reaching up to 5.6 a.i. kg/ha (Giesy et al. 2000). Consequently, it is not surprising that elevated glyphosate levels are present in these countries' water bodies (Annett et al. 2014). Previous studies have reported substantial concentrations of glyphosate in surface water, such as 0.7 $mg \times L^{-1}$ by Peruzzo et al. (2008) and 10.9 $mg \times L^{-1}$ by Ronco (2010), particularly in the surroundings of cultivation areas in the Pampas region of Argentina. Furthermore, other authors have reported lower PMG levels but frequent occurrences, pointing to PMG as a "pseudo-persistent" pollutant due to its continuous introduction into the environment under real agricultural practices (Primost et al. 2017).

Once in the aquatic environments, glyphosate can potentially impact non-target organisms, including fish species. PMG typically showed low acute lethal toxicity to freshwater fish (LC_{50}−96 h > 100 $mg \times L^{-1}$) (Pérez et al. 2011). However, numerous studies have reported that formulations containing the a.i. with co-adjuvants can markedly increase toxicity in fish (Giesy et al. 2000). Even more, sublethal effects (primarily of glyphosate-based herbicides but also of the active ingredient) have been reported in several fish species (Pérez et al. 2011; Gill et al. 2018). In previous studies, we determined neurotoxic effects in freshwater fish species exposed to the active ingredient (Menéndez-Helman et al. 2012). Additionally, alterations in subcellular energy balance and negative effects on the ultrastructure of the gills of *Odontesthes bonariensis* specimens exposed to a glyphosate-based herbicide (Menéndez-Helman et al. 2015; Menéndez-Helman et al. 2020).

The chemical characteristics of a contaminant and its release frequency into the environment are crucial factors in determining the degree of exposure of non-target species. Therefore, these factors must be considered when selecting the most appropriate ecotoxicity test. The use of partial life cycle tests, specifically focusing on reproduction and development, is suitable for assessing the effects of pollutants on fish species when the release frequency into the environment is either seasonal or continuous.

The pejerrey fish (*Odontesthes bonariensis*) belongs to the Atherinidae family and is a native species inhabiting lagoons in Buenos Aires Province, Argentina. This fish species holds socioeconomic importance for commercial and sport fishing, and it is also considered a promising candidate for aquaculture (Somoza et al. 2008). In lethal toxicity studies using pejerrey as a test organism, relatively low LC_{50}−96 h values have been estimated for various heavy metals, indicating the sensitivity of this species to environmental pollutants. Furthermore, an LC_{50}−96 h value of 147.5 $mg \times L^{-1}$ for PMG has been reported for this species (Carriquiriborde and Ronco 2006).

The pejerrey fish undergo external fertilization and indirect development, making the quality of sperm crucial for successful fertilization and reproductive processes. Several studies have indicated that both the percentage of motile sperm and sperm velocity are associated with reproductive success in different fish species, and reduction of these parameters can affect their fertility (Fauvel et al. 2010). Therefore, sperm motility can serve as an early and sensitive biomarker of aquatic pollution. For instance, there are reports of adverse effects on sperm motility in different species of fish, including pejerrey, due to exposure to metals, even at very low concentrations, as well as exposure to different organic compounds (Gárriz et al. 2015; Gallego and Asturiano 2018; Gárriz and Miranda 2020). For these reasons, an accurate analysis of sperm quality is necessary to obtain quantitative data and to compare the results appropriately. In recent years, the computer-assisted sperm analysis (CASA) system appeared as a promising tool for quantitative evaluation of sperm motility. It enables precise analysis of sperm sample quality in ecotoxicological studies (Wilson-Leedy and Ingermann 2007).

The use of fish embryos for ecotoxicological studies represents a promising model, enabling small-scale trials that can contribute to understanding the mechanism of the toxic compounds during the sensitive embryonic development stage (Embry et al. 2010). Additionally, larval tests can serve as a complementary tool to provide an integrated view of the toxic effects on development. In the case of pejerrey, the physio-morphological characteristics of the embryonic and larval development have already been described (Chalde et al. 2011).

The impacts of several anthropic contaminants identified in Pampas Lakes on the development and reproduction of pejerrey fish have been previously reviewed (Miranda and Somoza 2022). However, it is worth mentioning that there are currently no reports on the effects of glyphosate specifically on the sperm quality and early development. In this context, this study aimed to investigate the potential impact of glyphosate herbicide exposure, both in its active ingredient form and as a formulated product, on sperm motility, fertilization, and embryo-larval survival of pejerrey fish (*Odontesthes bonariensis*).

Materials and methods

Chemicals and treatment solutions

The glyphosate-based herbicide used in the study was Glifosato II Atanor®, acquired from a commercial Argentinean retailer. The formulation tested contained glyphosate

monopotassium salt at a concentration of 43.8 g/100 mL (equivalent to 35.6 g PMG/100 mL). Adjuvants are not specified in the label. The active ingredient (a.i., PMG 95%) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

The PMG doses (a.i. and glyphosate-based herbicide) were 0 (control), 1, 5, 10, or 50 mg PMG $\times L^{-1}$ expressed as glyphosate acid equivalent. The treatment solutions were prepared using dechlorinated tap water for sperm quality assays and embryo-larval survival assays, or using ground water for fertilization assays.

In all cases, the pH was adjusted to correspond to that of the control group, allowing for the distinction of the effect of PMG beyond its acidic characteristic.

Analysis of sperm quality

Sexually mature pejerrey males were selected from indoor tanks (3000 L) located at the aquaculture facilities of the *Instituto Tecnológico de Chascomús* (*Chascomús, Buenos Aires, Argentina*). Prior to handling, fish were anesthetized by immersion in a 100 mg $\times L^{-1}$ benzocaine solution for approximately 5 min. Subsequently, total sperm was stripped from each male, by abdominal massage, collected with a syringe, drawn into a capped tube, and kept on ice until further use. Any samples contaminated (urine or blood) were discarded. Only aliquots with higher motility indexes (Strüssmann et al. 1994) were used for motility assessment and fertilization assays. For video recording purposes, 1 µL of semen was activated by mixing with 1800 µL of each treatment solution at room temperature (22 °C). Following gentle stirring, 10 µL of this dilution was immediately loaded into a Neubauer chamber and covered with a coverslip (24 mm × 24 mm), previously pre-coated with 1% PVA solution and dried at 60 °C to avoid the adhesion of spermatozoa (Chalde et al. 2016). Videos of each sperm sample were captured using a Basler 602fc camera (Ahrensburg, Germany) mounted on a trinocular Olympus CX 41 microscope at × 10 magnification. Recordings lasted for 35 s at a rate of 100 frames/s in.avi format using the AMCAP software (Basler Vision Technologies, Ahrensburg, Germany). Subsequently, the videos were edited using VIRTUALDUB-1.9.0 software (virtualdub.org) and exported as sequences of.jpg images. These images, corresponding to 1 s of video, were further processed using IMAGEJ software (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) and analyzed using the CASA (computer-assisted sperm analyzer) application (University of California and Howard Hughes Medical Institute, USA). Specifically, the sequences corresponding to the seconds 10 and 30 post-activation (pa) were analyzed. Parameters such as motility (%), VCL (curvilinear velocity), VAP (average path velocity), and VSL (straight line velocity) were assessed using the same configuration of the CASA system as described in Chalde et al. (2016). Each sperm sample was subjected to duplicate testing for each treatment, with biological replicates ($n = 4–5$).

Fertilization assays

In spring, during the spawning season, eggs from three mature females reared in captivity were stripped. In vitro fertilizations were conducted with around 100 eggs for each treatment. These were fertilized using a sperm pool of 7–10 males per egg set, activated with the following solutions: water (control), 1, 5, 10, or 50 mg PMG $\times L^{-1}$ (a.i or formulated) by duplicate. For activation, 10 µL of semen was mixed with 15 mL of each treatment solution at the specified concentrations, the tube was inverted three times, and then solution was immediately added to the eggs, approximately 3 s post-activation. Egg samples were incubated at 24 °C for 24 h. Fertilization % was assessed, according to the procedures previously described by Chalde et al. (2011).

Analysis of embryo-larval survival

Early-stage embryos, without ocular pigments, corresponding to embryo stages 47–52 h post-fertilization, at the beginning of neurulation (Chalde et al. 2011), were obtained from the broodstocks kept at the aquaculture facilities of the "Instituto Tecnológico de Chascomús." The embryos were separated into groups of 10 and were placed in plastic recipients. The concentrations used for these assays were the same as mentioned in the previous sections. The solutions were prepared initially, simulating a single contamination event, and the complete volume of each solution (30 mL) was changed daily, at the same time with the corresponding solution of each treatment, for a total of 6 days. The renewals were carried out with control water for all treatments from this moment (1 day after cephalic pigmentation was observed). Embryo survival and hatched success were monitored daily, and dead organisms were removed.

Larvae 1–2 days post-hatching (from embryos without previous exposure) were exposed to the same herbicide concentrations (a.i. and formulated). The point of no return (PNR = days at which 50% of starved larvae died) was assessed. The experiments conducted under PNR conditions (without feeding, water renewal, or aeration) were performed in a programmable incubator (Bio-Control AX-Z) with controlled temperature (19.5 ± 0.5 °C) and photoperiod (12L:12D), and all treatments were assayed in triplicate.

Analytical concentration of PMG

PMG concentrations in the exposure aqueous media were determined by ion chromatography (Zhu et al. 1999). A

Dionex DX-100 chromatograph equipped with a conductivity detector, Dionex AG-4 and AS-4 as analytical columns, and a 25 µL sample loop was used for this purpose: eluent, $NaOH/Na_2CO_3$ (4 mM/9 mM), and flow rate, 1 $mL \times min^{-1}$. Data acquisition was conducted using the Clarity Lite software. Samples from each assay (nominal concentrations: 0, 1, 5, 10, and 50 mg $PMG \times L^{-1}$) and standards freshly prepared were injected.

Statistical analysis

Two recorded video sequences were analyzed and averaged for each sperm sample to obtain a single value per parameter per fish. For sperm quality analysis ($n = 4$–5), fertilization % ($n = 3$), embryo survival ($n = 3$), and larval survival (PNR) ($n = 3$), the results are presented as mean ± SEM for the control and each exposed group. The embryo survival (%) and hatching rate (%) versus time were calculated for each group. The cumulative mortality versus time and the time of PNR were assessed for the analysis of the potential effects on the survival of larvae.

Statistical comparisons between the exposed groups and the control for each parameter (motility parameters, fertilization rate, and PNR) were conducted using the Kruskal–Wallis non-parametric ANOVA, followed by Dunn's multiple comparison test (GraphPad Prism Software). Statistical analysis was performed by two-way ANOVA (treatment × time) followed by post hoc Bonferroni test for the analysis of embryo survival. Statistical significance was established at $p < 0.05$.

Results

Sperm quality

The CASA parameters for the control group were 49.9 ± 5.7% (mean ± SEM) of motile sperm, VCL = 131.3 ± 4.9 µm/s, VAP = 94.2 ± 4.3 µm/s, and VSL = 67.7 ± 5.1 µm/s, at the initial time (second 10 pa). These values are similar to those previously described for *O. bonariensis* (Gárriz et al. 2015; Gárriz and Miranda 2020). As it is known, all these sperm quality parameters significantly decrease during the first minute post-activation. This decrease was 22.7%, 89.1 µm/s, 43.6 µm/s, and 35.5 µm/s for sperm motility, VCL, VAP, and VSL at second 30 pa, respectively.

The glyphosate-based herbicide exposed groups showed an increasing trend in motility % at the initial time, but statistically significant differences were observed only for the highest concentration tested compared to the control group (Fig. 1A). No differences were determined for the other motility parameters (Fig. 1B–D). Similar profiles were observed for formulated exposure at second 30 pa than at the initial time. On the other hand, the active ingredient showed a declining trend in motility percentage for some of the treatments, with this decrease being statistically significant only for the highest concentration at second 30 pa. Additionally, for this treatment, a statistically significant decrease in other motility parameters (VAP and VSL) was also observed (Fig. 2A–D).

Fertilization assay

The glyphosate-exposed groups did not show statistically significant differences in fertilization rate (%) when compared to the control group (76 ± 13%, Fig. 3).

Embryo-larval survival

Embryo survival until hatching ranged from 80 to 97% under control conditions (maintained in tap water, at controlled temperature (19.5 ± 0.5 °C) and photoperiod (12L:12D)). The development of exposed embryos did not exhibit relevant differences compared to the control group. Only minor statistically significant differences in survival (%) were observed in the group exposed to the highest concentration of PMG (a.i.) (Fig. 4). There were no effects in the morphology or temporal evolution (acceleration or retardation) of the embryonic stages, nor in the hatching rate over time (Fig. 5).

In the larvae exposure assays to the active ingredient, we did not observe any effect on its mortality (Fig. 6A). However, when exposed to the formulated product, we found a significant adverse effect, with larvae reaching a mortality rate of 100% within a few hours at the highest concentration (Fig. 6B). The statistical comparison between the curves is evident from the estimated PNR values (Table 1). This value was statistically lower (< 1 day) for the highest concentration of glyphosate-based herbicide than the respective control group.

Finally, it should be noted that analytical concentrations of glyphosate in the media of the different treatments were determined in all assays (Supplementary Tables S1, S2, S3, and S4). This analysis confirmed that the analytical concentrations were similar to the nominal ones.

Discussion

The use of pesticides has continued to increase over the last few decades in the South America region. Among these chemicals, glyphosate stands out as one of the most widely employed herbicides worldwide. Its ubiquity in agricultural practices raises concerns regarding its potential impacts on aquatic organisms, particularly freshwater fish. Therefore, elucidating the specific effects of glyphosate on the reproduction and development of a native freshwater fish, such

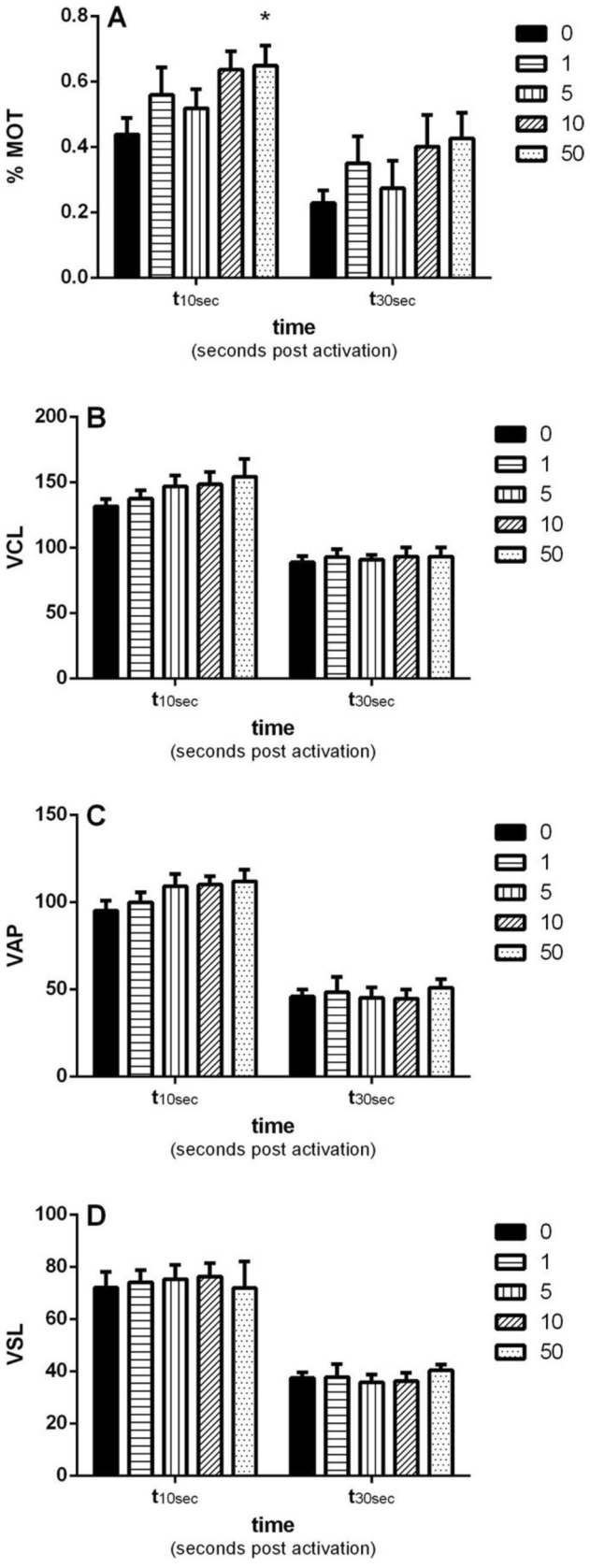

Fig. 1 Sperm quality analyzed by CASA of an activated sample with control medium (tap water) or in the presence of different concentrations of glyphosate-based herbicide (formulated): 1, 5, 10, and 50 mg PMG × L^{-1}. Motility percentage is plotted at different times after activation (**A**), VCL (**B**), VAP (**C**), and VSL (**D**)

as pejerrey (*Odontesthes bonariensis*), carries considerable importance in addressing the broader implications of pesticide pollution in freshwater environments.

Numerous studies have highlighted the significance of investigating sperm motility in externally fertilizing animals, as it is pivotal for reproductive success. In such species, gametes come into direct contact with the external environment during the fertilization process. Sperm motility plays a crucial role in the encounter between sperm and eggs, being a determining factor in successful fertilization (Bobe and Labbé 2010).

Our results demonstrated a statistically significant increase in the percentage of motile sperm at the initial time point for the highest concentration of the glyphosate-based herbicide tested. A similar trend is observed for this treatment and exposure to lower concentrations for both evaluated time points. This effect could be attributed to the presence of adjuvants that increase membrane permeability, allowing for a faster osmotic change that triggers earlier activation of motility. This hypothesis is supported by the absence of an effect of increasing the percentage of motility after exposure to glyphosate as an active ingredient unlike what was observed with the glyphosate-based herbicide.

The observed increase in sperm motility could have significant implications for the reproductive success of pejerrey fish. Enhanced sperm motility at the initial stages of fertilization may facilitate greater fertilization rates and subsequent embryonic development. However, it is essential to consider the potential trade-offs associated with increased sperm motility, as excessive activation may lead to premature exhaustion of sperm reserves or decreased sperm quality over time. For example, Lugowska (2018; 2020) exposed fish sperm to a wide range of glyphosate-based herbicide Roundup concentrations (0.1 to 50 mg × L^{-1}) and reported a significant reduction in motility duration at 20 mg × L^{-1} in common carp and at most concentration in grass carp. In the present work, the duration of sperm motility was not evaluated, so it cannot be ruled out that the effect of exposure to the glyphosate-based herbicide may lead to a decrease in motility over time.

In contrast to the glyphosate-based herbicide effects, exposure to the active ingredient resulted in a statistically significant decrease in parameters such as motility percentage, VAP, and VSL for the highest concentration tested.

Previous studies have described the effects of glyphosate on sperm viability and motility in various fish species (review in Lopes et al. 2022). Reduced sperm quality,

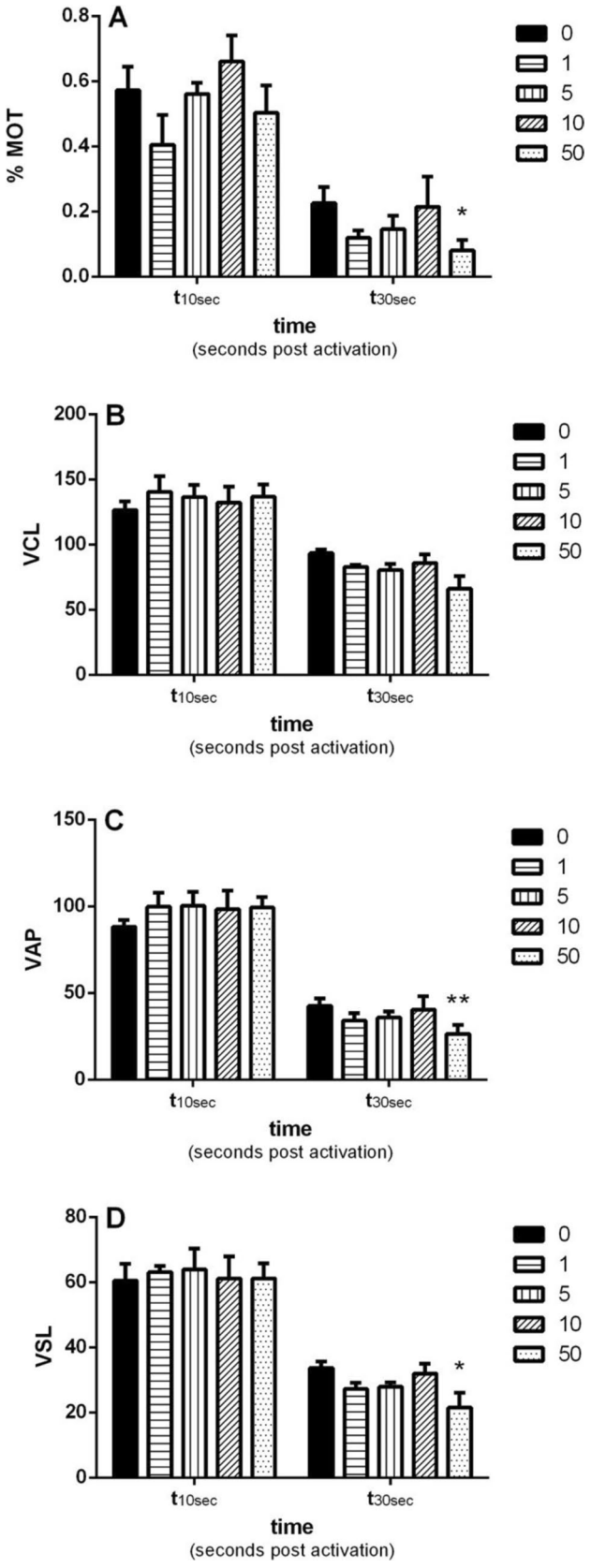

Fig. 2 Sperm quality analyzed by CASA of an activated sample with control medium (tap water) or in the presence of different concentrations of glyphosate (a.i.): 1, 5, 10, and 50 mg PMG × L^{-1}. Motility percentage is plotted at different times after activation (**A**), VCL (**B**), VAP (**C**), and VSL (**D**)

including compromised sperm plasma membrane integrity, mitochondrial functionality, DNA integrity, and sperm motility, has been reported in male guppies (*Poecilia vivipara*) following exposure (96 h) to Roundup® at concentrations of 0.13 and 0.70 mg × L^{-1} of glyphosate (Harayashiki et al. 2013). Similar results were determined in *Jenynsia multidentata* following acute exposures (96 h) to different glyphosate formulations (Sánchez et al. 2017). Additionally, reductions in sperm motility and motility period were observed in zebrafish (*Danio rerio*) following acute exposures to 5 and 10 mg × L^{-1} of the active ingredient, with concomitant diminishment in mitochondrial functionality, membrane integrity, and DNA integrity at the highest concentration (Lopes et al. 2014). These effects observed after in vivo exposure of males could be due to a direct effect on the sperm cells or may result from other pathways, such as an alteration in spermatogenesis.

Furthermore, some studies have determined impairments due to exposure in vitro of gametes in the activation medium. Exposure to formulations containing 50 µg × L^{-1} of the active ingredient, a concentration within legal limits in US waterbodies, resulted in an adverse effect on sperm quality of *Astyanax lacustris* (Gonçalves et al. 2018). Comparable results, including DNA fragmentation and motility reduction, were obtained in rainbow trout when exposing sperm to glyphosate as the active ingredient (2.5, 5, 10 mg × L^{-1}) (Akça et al. 2021). Similarly, it was determined that exposure to the herbicide Roundup affected sperm motility and mitochondrial integrity in human sperm cells (Anifandis et al. 2018).

The reduction in sperm motility and velocity parameters in response to glyphosate exposure suggests potential adverse effects on the reproductive fitness of pejerrey fish. Decreased sperm motility can impair the ability of sperm to reach and fertilize eggs, thereby compromising reproductive success and potentially leading to population decline (Devaux et al. 2015). Interestingly, our findings suggest that the adjuvants present in the glyphosate formulation may play a crucial role in modulating sperm motility, rather than glyphosate itself. It is worth noting that there is a lack of previous studies comparing the effects of the active ingredient and the formulated product on sperm motility of fish species. The results from this study underscore the importance of considering the entire formulation when assessing the effects of herbicides on aquatic organisms. Furthermore, concerning the results of motility, although the statistically significant effects observed are associated with a concentration

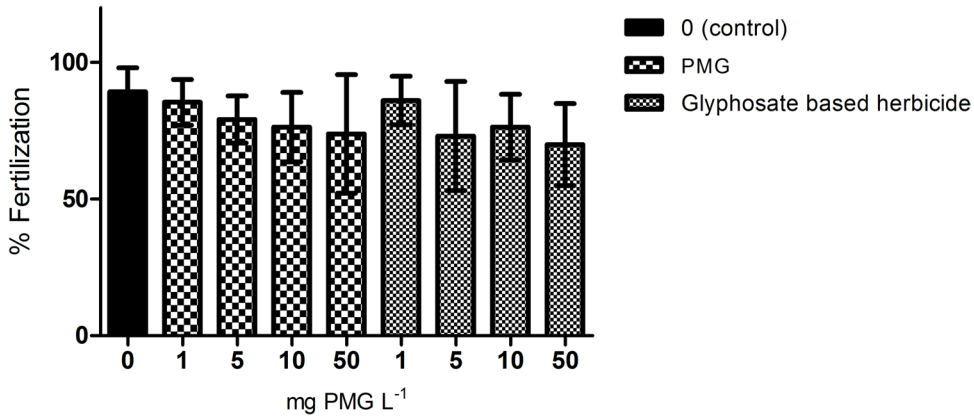

Fig. 3 Fertilization rate of coincubations (sperm-eggs) carried out in control medium or in the presence of different concentrations of glyphosate (PMG a.i. or formulated) (1, 5, 10, and 50 mg PMG × L^{-1})

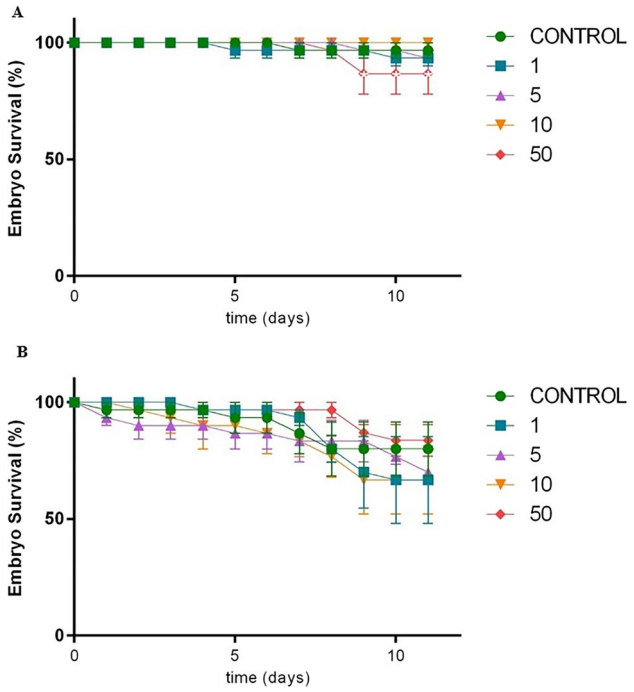

Fig. 4 Embryo survival (%) of pejerrey fish (*O. bonariensis*) exposed to different concentrations of glyphosate (1, 5, 10, and 50 mg PMG × L^{-1}) or maintained in control conditions. **A** PMG (a.i.) or **B** glyphosate-based herbicide (formulated). Values are means ± SEM ($n = 3$). The asterisks indicate significant differences with the control curve for each time ($p < 0.05$)

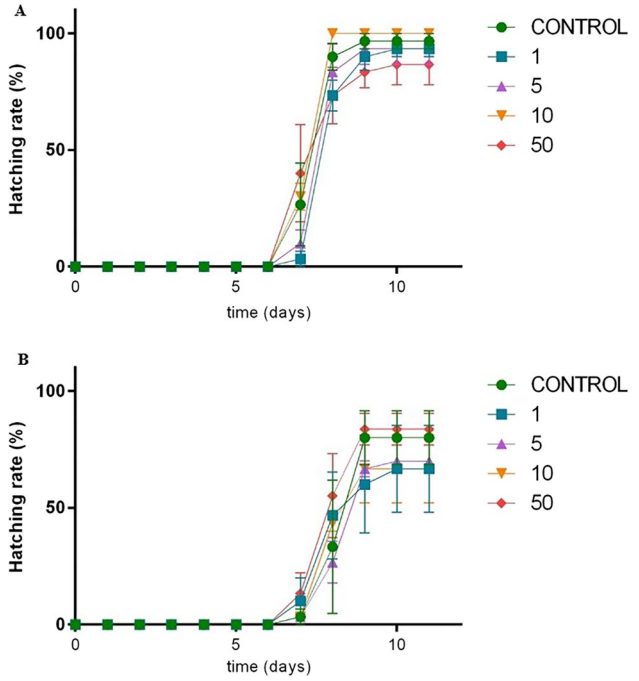

Fig. 5 Hatching rate (%) of pejerrey fish (*O. bonariensis*) exposed to different concentrations of glyphosate (1, 5, 10, and 50 mg PMG × L^{-1}) or maintained in control conditions. **A** PMG (a.i.) or **B** glyphosate-based herbicide (formulated). Values are means ± SEM ($n = 3$)

higher than those typically found in the environment, it is noteworthy that certain trends are observed even at lower concentrations that are environmentally relevant.

The results of the fertilization assay did not show any statistically significant differences. However, for both the formulated product and the active ingredient exposures, a decreasing trend in the fertilization percentage was observed, suggesting that the results cannot be conclusive. Studies on the effects of glyphosate on fertilization in fish are scarce. Uren Webster et al. (2014) reported that exposure of breeding zebrafish (*Danio rerio*) to Roundup at 10 mg a.i. × L^{-1} and glyphosate at 10 mg × L^{-1} reduced egg production but not the fertilization rate.

Regarding exposure assays during embryonic-larval stages, we observed a clear difference in susceptibility. No relevant differences were found in the embryonic development of exposed groups compared to the control. No effects were observed in the morphology or temporal evolution of the embryonic stages, nor in the hatching rate over time. Only a minor but statistically significant decrease in survival was noted in the group exposed to the highest

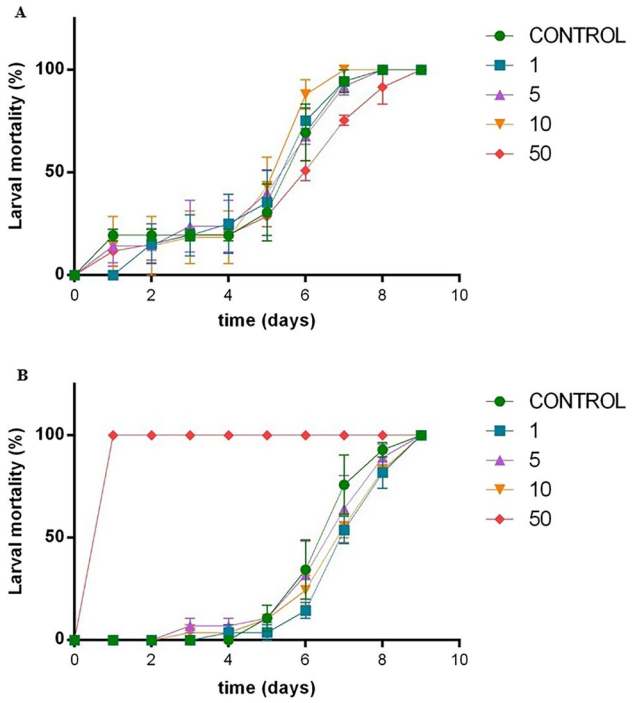

Fig. 6 Larval mortality (%) of pejerrey fish (*O. bonariensis*) maintained in control water or exposed to different concentrations of glyphosate (1, 5, 10, and 50 mg PMG × L^{-1}). **A** PMG (a.i.) or **B** glyphosate-based herbicide (formulated). Values are means ± SEM ($n = 3$)

Table 1 PNR of hatched larvae exposed to different concentrations of glyphosate (PMG, a.i., or F, formulated) or maintained in control water. *$p < 0.05$ vs control, $n = 3$

Glyphosate exposure	PNR (days)	
(Nominal, mg PMG × L^{-1})	*F*	*PMG*
0 (control)	6.32 ± 1.02	5.33 ± 1.04
1.0	6.93 ± 1.01	5.07 ± 1.04
5.0	6.50 ± 1.02	5.02 ± 1.05
10.0	6.76 ± 1.01	4.98 ± 1.09
50.0	~0.0*	5.67 ± 1.06

concentration of PMG (a.i.). However, larvae exhibited clear susceptibility to the formulated product, with 100% mortality observed at the highest concentration during the initial hours of exposure. The estimated PNR value for this treatment was < 1 day, statistically lower than the 6 days of the respective control group. The differential susceptibility of embryos and larvae is consistent with findings from previous studies suggesting that larvae are more sensitive than embryos to a wide range of substances, as indicated by the NOEC (no observed effect concentration) data (Hutchinson et al. 1998).

Several authors have documented the effects of glyphosate and its formulations on the embryonic development of certain fish species (review in Lopes et al. 2022). Alterations in hatching time (retardation or premature), as well as a decrease in the number of hatched eggs, have been described in different species exposed to the active ingredient and glyphosate formulations during embryonic development (Yusof et al. 2014; de Brito Rodrigues et al. 2017; Zhang et al. 2017; Fiorino et al. 2018; Smith et al. 2019). Conversely, no differences were observed in the hatching percentage in our experiments, even for the highest concentrations tested, whether of the active ingredient or the formulated product. Additionally, Zebral et al. (2017) determined that exposure to 5.43 mg acid equivalent × L^{-1} Roundup Transorb R increased mortality in the embryonic development of pejerrey, *Odontesthes humensis*. Notably, other studies found consistent results with our experiments. No adverse effects on survival during embryonic development in rainbow trout exposed to glyphosate formulation (0.1 to 1 mg × L^{-1}) (Weeks Santos et al. 2019) and zebrafish embryos exposed to the active ingredient (3 µg × L^{-1} to 10 mg × L^{-1}) (Stehr et al. 2009) were reported. Likewise, Uren Webster et al. (2014) found a higher mortality in embryos from exposed parents' populations to the formulated product or a.i. (10 mg × L^{-1}), but they did not observe any effect on embryonic development when analyzing the direct effect of exposing control embryos.

Regarding the larvae stage, numerous studies have described behavioral effects. It has been observed that glyphosate impairs locomotor activity and causes deficits in motor coordination and sensory ability (Lopes et al. 2022). Additionally, other sublethal effects such as neurotoxicity, oxidative stress effects, and malformations have been described (Díaz-Martín et al. 2021; Lopes et al. 2022). Lugowska (2018) determined that Roundup affected the quality of newly hatched larvae from exposed embryos of common carp by increasing their mortality. In many experimental designs, exposures are initiated during the embryonic stage, potentially leading to teratogenic impairments that may only become apparent after hatching. Similarly, in our study, we cannot rule out the possibility that hatched larvae from exposed embryos may exhibit impairments post-hatch.

One hypothesis is that the observed results may be due to a differential susceptibility of embryos and larvae to herbicide exposure, which could be related to the impermeability of the chorionic membrane of fish embryos to various contaminants. In this sense, a hard chorion was previously described for pejerrey embryos (Macoretta and Miranda 2020). The chorionic membrane acts as a protective barrier, limiting the uptake of external substances and reducing the potential for toxic effects during embryonic development. In contrast, larvae lack this protective barrier and are thus

more susceptible to the toxic effects of herbicides, which may explain the higher mortality rates observed in our study.

In summary, our findings reveal glyphosate's intricate impacts on aquatic organisms, affecting key processes such as sperm motility, fertilization, and development. Future research should explore the variability between the active ingredient and the formulated product, delve into the specific influence of adjuvants on sperm motility, assess longer exposure times and lower concentrations, and investigate the lower susceptibility of embryos compared to larvae.

Conclusions

Our study provides novel insights into the effects of glyphosate on the reproduction and development of an Argentinian native freshwater fish species, pejerrey fish (*Odontesthes bonariensis*). The comparative analysis of active ingredient and glyphosate-based herbicide on sperm motility is a noteworthy aspect of our research. While sperm motility (%) increased in response to certain concentrations of the formulated product, exposure to the active ingredient resulted in significant negative effects on various quality sperm parameters. Furthermore, we observed distinct responses depending on the developmental stage and type of herbicide exposure (active ingredient or formulated product). The results underscore the greater susceptibility of larvae compared to embryos, highlighting the importance of considering different developmental stages when assessing the effects of environmental pollutants. Overall, our findings emphasize the complexity of glyphosate impacts on freshwater aquatic organisms.

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Author contribution LAM and RJMH contributed to the study conception and design. Material preparation, data collection, and investigation were performed by AG, RJMH, and LAM. Formal analysis was conducted by RJMH and MCRM. Funding acquisition: LAM and RJMH. Resources: LAM and MCRM. The first draft of the manuscript was written by RJMH and LAM, and AG and MCRM commented on previous versions of the manuscript.

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Data availability Available data were deposited in CONICET repository: https://www.ri.conicet.gov.ar/.

Declarations

Ethical approval All embryos, larvae, and adult fish were handled according to the manual of the Universities Federation for Animal Welfare Use and Care Committee on the Care and Handling of Laboratory Animals and according to the local regulations of San Martín University.

Consent for publication Not applicable.

Consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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