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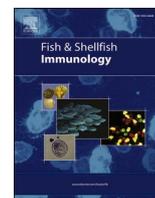
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## Changes in defense capacity to infectious hematopoietic necrosis virus (IHNV) in rainbow trout intergenerationally exposed to glyphosate

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### ABSTRACT

Glyphosate, the most widely used herbicide active substance worldwide, has raised many scientific, political and public debates in the context of its recent re-registration in the European Union, highlighting in particular a lack of data concerning its potential generational effects. In this study, we investigated the intergenerational toxicity of this active substance used alone or coformulated in glyphosate-based herbicides (GBHs) on the ability of rainbow trout (*Oncorhynchus mykiss*) to face a viral challenge. Juvenile trout from parents exposed for eight months to four different chemical exposure conditions (non-exposed control, pure glyphosate, Roundup Innovert®, and Viaglif Jardin®) were experimentally infected with the infectious hematopoietic necrosis virus (IHNV). Various enzymatic and hemato-immunological markers were assessed before and after the viral challenge. Chemical contamination with GBHs strongly modulated viral trout susceptibility. Pure glyphosate induced a cumulative mortality of 35.8%, comparable to the control (37.0%), which was significantly reduced with Roundup Innovert® (−9.9%) and increased (+14.8%) with Viaglif Jardin®. No modification was observed for the biomarkers analysed for any conditions. These results demonstrate that the nature of the co-formulants associated to glyphosate in GBHs can modulate the susceptibility of fish to pathogens.

### 1. Introduction

Glyphosate is an herbicidal active substance (AS) widely used in agriculture for its effectiveness in controlling weed development. This chemical compound, associated with co-formulants in the form of “glyphosate-based herbicides” (GBHs), has raised health and environmental concerns because of its ubiquity in the environment, including in surface water. Moreover, divergent risk assessment strategies among health and environment agencies have complicated its re-registration in the European Union [27]. Different toxic effects of both the AS and GBHs have been identified in fish, such as oxidative stress and metabolic impairments [2]. However, although some immunotoxic effects have been detected [7,9,10,19,20,24], the immunomodulating properties of glyphosate are still poorly understood. Only a few studies have reported the use of integrative tools such as challenges with pathogens, despite their proven relevance [21]. Higher susceptibility to the bacterium *Aeromonas hydrophila* has been induced by acute exposure of silver catfish (*Rhamdia quelen*) juveniles to 730 µg L<sup>−1</sup> of a GBH [11]. Studies

have indicated that parental exposure to glyphosate, whether co-formulated or not, could induce developmental impairments, possibly associated with epigenetic changes [26], and could increase offspring susceptibility to recontamination by the parental compound [29]. However, the impact of this mode of exposure on glyphosate immunotoxicity has not yet been studied. Nevertheless, impairments of the immune system related to intergenerational exposure to other chemical contaminants have already been identified [6,14,31].

In the present study, we investigated changes in the overall defense capacity of rainbow trout following chronic exposure to an environmental low dose of glyphosate as the active substance or in the form of two different GBHs. Different resistant profiles were observed using an original approach consisting in submitting F1 generation fish derived from F0 genitors previously exposed to the chemical compounds to a viral challenge with the hematopoietic necrosis virus (IHNV). In addition to survival, various biochemical parameters were assessed before and after the infection to identify metabolic modulation, generation of oxidative stress, and alterations in immune defenses.

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## 2. Materials and methods

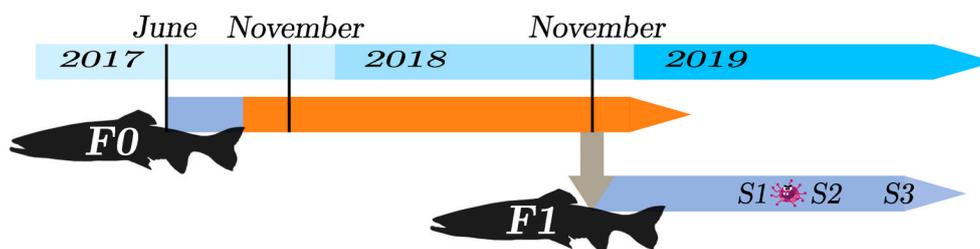
Fish experiments were conducted using specific pathogen-free (SPF) rainbow trout reared in the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France). Fish experimentation was carried out in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). Fish were maintained in tanks containing river water filtered with sand filter (approximately 20  $\mu\text{m}$ ), with a water flow rate to ensure complete renewal once an hour and maintain appropriate physico-chemical conditions and oxygen saturation greater than 60%. Our experimental facilities are supplied with river water with a mean pH of 7.8, a conductivity of 400  $\mu\text{S m}^{-1}$  and a hardness of 12°TH. Concentrations of ammonia, nitrate and nitrite are close to 0. Physico-chemical analyses of the water were regularly carried out to guarantee excellent maintenance conditions for all the fish. A photoperiod of 12 h of daylight was maintained throughout the experiments. Different batches of an F1-2018 generation of SPF rainbow trout were produced from a non-contaminated (C; control) generation and three chemically contaminated F0-2014 generations exposed for 8 months to 1  $\mu\text{g L}^{-1}$  of glyphosate (see Fig. 1 for graphical explanation and the previous article of Le Du-Carrée et al. [12] for more details on the chemical exposure procedure) administered as the pure active substance (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6) or two GBHs, Roundup Innovert® (R; Agrilisa - for professional use) and Viaglif Jardin® (V; Agrilisa - for home gardens). Once the genitors have reached sexual maturity, reproduction was done as already described (see Le Du-Carrée et al. [12]) and embryonic development until the eyed stage (see Bobe et al. [4]) was conducted in 300 L tanks continuously renewed with river water (filtered with sand and cotton wool filters) with a flow rate of approximately 300 L h<sup>-1</sup>. After the eyed stage period, embryos were placed in 40 L tanks positioned in a confined room. Temperature during embryonic development was maintained at 8 °C  $\pm$  2 °C. After this stage, water temperature varied from 6 to 15 °C. A trout-specific feed (Le Gouessant®), adapted to the fish size, was given ad libitum. After approximately 9 months of development without chemical exposure to assess only the intergenerational effect, 180 juveniles (weight and size, mean  $\pm$  standard error, of 74.0 g  $\pm$  4.08 g and 14.2 cm  $\pm$  0.30 cm, respectively) of each chemical exposure condition divided in three 50 L tanks were challenged by contact with an infectious dose of 10<sup>4</sup> TCID<sub>50</sub> mL<sup>-1</sup> *IHNv* for 3 h in a reduced volume of hyperoxygenated river water at 11 °C  $\pm$  2 °C. Uninfected control tanks (60 fish of each chemical exposure condition) were similarly challenged with non-infected *epithelioma papulosum cyprini* cell supernatant [15]. General behavior of the fish, specific clinical signs of rhabdoviruses infection, and mortality were recorded twice a day for 9 weeks. At the mortality peak, viral presence was checked. Detection of viral genome was assessed in a pool of organs (brain, spleen, kidneys and heart) from dead fish in each test condition using specific RTqPCR [3]. Sampling was performed before

the viral infection (S1), 4 days post-infection (dpi; S2) and 63 dpi (S3). Euthanasia was done with addition of a lethal dose of 100 ppm of Eugenol into the tank water. The animals were put in contact with the product until complete disappearance of all respiratory activity. Blood was drawn from the caudal vein into lithium heparinized vacutainer tubes to calculate white blood cell (WBC) and red blood cells (RBC) counts at S1 and S2 [8], and to semi-quantify anti-*IHNv* antibodies in survivors at S3 (see Louboutin et al. [15] and Ananda Raja et al. [1] for method reference). The liver and brain were sampled at S1 to analyze oxidative stress (superoxide dismutase, SOD; glutathione peroxidase, GPx), neuromediator-related enzyme (acetylcholine esterase, AChE) and energy metabolism markers (citrate synthase, CS; cytochrome-c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G6PDH); see Le Du-Carrée et al. [12] for detailed information on the assays. Spleens sampled at S2 were used to quantify the concentration of immune markers (fish interleukin 1 $\beta$  (IL - 1 $\beta$ ) and fish tumor necrosis factor  $\alpha$  (TNF -  $\alpha$ ) assayed with Biorbyt® ELISA kits).

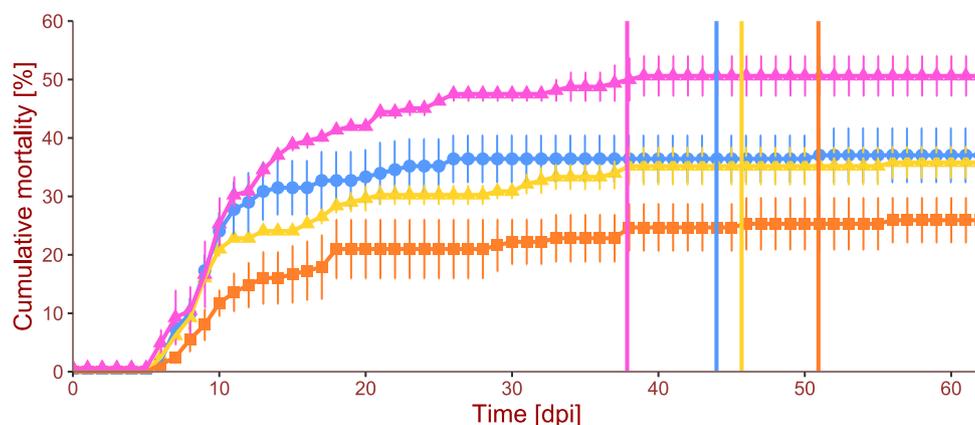
## 3. Results

After nine months of development and before the viral challenge, enzymes representing energetic metabolism (i.e. CS, CCO, LDH, and G6PDH), as well as AChE, and SOD activities were not altered by the parental chemical exposure in the juvenile offspring compared to the control (data not shown). GPx activity was the only parameter affected. Mean values were comprised between 0.23  $\pm$  0.0063 and 0.25  $\pm$  0.010 IUmg<sup>-1</sup> for the control and conditions exposed to the two GBHs, while a mean value of 0.20  $\pm$  0.0083 IUmg<sup>-1</sup> was detected for the glyphosate condition (*p*.value = 0.04, d.f. = 3 and *f* = 3.08). A significant decrease in GPx activity in F1 fish exposed to glyphosate compared to the control was detected by a post-hoc test (-13%; *p* < 0.05).

The mortality kinetics induced by *IHNv* infection (see Fig. 2) were different depending on the conditions, and the cumulative mortality rates were shown to be significantly affected by chemical contamination (*p*.value = 0.000 3, d.f. = 4 and  $\chi^2$  = 21.40). Cumulative mortality reached 37%, 36%, 26% and 51% for the control, glyphosate, Roundup and Viaglif conditions, respectively. Target organs of infected fish that died during the peak of mortality were detected positive for *IHNv* by qPCR. Faster and higher mortality appeared to be observed in fish exposed to Viaglif, represented by shorter restricted mean survival times (RMST, an integrated parameter representing both the dynamics and the intensity of mortality [28]; mean  $\pm$  se, n = 3) of 51  $\pm$  2.3 dpi, compared to 44  $\pm$  2.4, 46  $\pm$  1.1 and 51  $\pm$  2.3dpi respectively, for the control and glyphosate conditions while fish exposed to Viaglif tended to present a lower RMST of 38  $\pm$  1.2. Logrank test revealed that the mortality rates for the Viaglif and Roundup conditions respectively, were significantly higher (+39% difference; *p*.value = 0.017) and lower (-29% difference; *p*.value = 0.031) compared to mortality rates of the control and glyphosate conditions. Proportions of *IHNv*-seropositive fish were between 73% and 100% among chemical treatments, with an overall mean of 1235. Approximately 60% highly seropositive fish (antibody titer greater than 640) were found, regardless of the chemical condition.



**Fig. 1.** Timeline of the study on generations F0 2014 and F1 2018. Dark blue and orange lines represent phases without and with chemical exposure, respectively. The exposure of F0 2014 lasted 8 months to a dose of 1  $\mu\text{g L}^{-1}$  of glyphosate. The viral challenge with *IHNv* is represented by a virus symbol. Sampling was performed one day before the viral infection (S1), four days post-infection (S2), and 63 days later (S3).



**Fig. 2.** Kinetics of cumulative mortality in F1 fish intergenerationally exposed to chemical contamination and infected at nine months of development with *IHNv*. Data are expressed as a function of time in days post-infection (dpi) for the G (in yellow), R (in orange), V (in purple) and control (in blue) groups. Error bars represent standard errors, vertical bars the restricted mean survival time.

WBC and RBC counts performed before or after the viral infection were not significantly different (data not shown). Differences in  $IL-1\beta$  concentrations were observed (Table 1;  $p$ -value = 0.002, d.f. = 3 and  $f$  = 11.52), with a reduction of 62% in fish intergenerationally exposed to glyphosate compared to the controls ( $p < 0.05$ ), while  $TNF-\alpha$  concentrations were similar among all the conditions.

#### 4. Discussion

Our experimental design was conceived to investigate the potential effects of glyphosate and GBHs on the defense capacity of an offspring born from adult rainbow trout exposed chronically to low, environmentally relevant doses of glyphosate as the active substance or in the form of two different GBHs. Results showed that neither the oxidative defense system nor energetic metabolism were affected by the chemical exposure of the F0 generation. This indicates that glyphosate may not have generated oxidative stress and therefore additional energy requirements, explaining the absence of observed metabolic readaptation. GPx activity, involved in the first line of defense against reactive oxygen species (ROS) [16], was the only activity found to be reduced in the context of intergenerational exposure to pure glyphosate. A similar observation, i.e. a decrease of antioxidant enzyme activities including GPx, was reported in the offspring of an F0 zebrafish (*Danio rerio*) generation exposed chronically to an algal toxin [14]. A higher concentration of glyphosate (i.e. ranging from 2.25 mg L<sup>-1</sup> to 15 mg L<sup>-1</sup>) also induced a reduction in antioxidant enzymes activities in *Pseudoplattystoma* sp directly exposed for 96 h to a GBH, and this was probably due to increased ROS levels [25]. While biochemical parameters measured in juveniles exposed intergenerationally to the chemicals did not indicate any physiological effects, the *IHNv* challenge revealed that only GBHs affected the ability of rainbow trout to survive the infection. This approach has proven to be particularly relevant to measure the effect of

parental exposure to chemicals on the overall defense capacities of the F1 generation. It showed that parental exposure to Viaglif generated higher mortality in the F1 juveniles while, interestingly, Roundup exposure increased the survival of the intergenerationally exposed fish. Kreutz et al. [11] have shown that a GBH could induce a higher susceptibility of silver catfish juveniles to the bacterium *Aeromonas hydrophila* and other authors have reported similar observations [5,6]. However, to our knowledge, our results are the first to demonstrate a decreased in fish susceptibility to a pathogen after exposure to glyphosate through a GBH. Nevertheless, in a review on fish immuno-ecotoxicology, Rehberger et al. [21] revealed that 17% of the experiments considering pathogen challenges detected lower mortality rates in fish exposed to different contaminants compared to the non-exposed controls. Parental exposure to Roundup might have stimulated the immune defenses of the fish, resulting in a greater ability to face the viral infection. The immune-modulating properties of glyphosate formulations we observed do not seem to be related to hematological changes because white and red blood cell counts revealed no effects of the intergenerational exposure. Quantification of two cytokines involved in antiviral immune response [22],  $TNF-\alpha$  and  $IL1-\beta$ , in the spleen, showed that parental exposure to pure glyphosate reduced levels of  $IL1-\beta$  but not  $TNF-\alpha$ . Direct GBH exposures have been reported to modify gene expression [13,17,23,30] and cytokine concentrations [18]. Liu et al. [14] also demonstrated that intergenerational exposure of zebrafish to an algal toxin reduced both  $IL1-\beta$  and  $TNF-\alpha$  gene expression. In this study, the variation of  $IL1-\beta$  expression observed in juveniles from parents exposed to glyphosate did not affect their susceptibility to the virus.

#### 5. Conclusions

This study provides evidence that parental exposure of rainbow trout to GBHs could modulate the defense capacity of juvenile trout to a viral challenge, while parental exposure to the pure active substance had no effect. Depending on the GBH formulations considered, survival following virus exposure was either increased or decreased. Based on the parameters measured, neither the disruptions in metabolic activity and the antioxidant defenses, nor impairment of the immune system can explain the observed differences in survival. Parental exposure to pure glyphosate induced only minor physiological changes by reducing the activity of an antioxidant enzyme and the concentration of a key cytokine of the immune system. Additional in-depth studies evaluating the generational impact of a low dose of both pure and co-formulated glyphosate are required to determine the mechanisms involved in the modulation of fish susceptibility to pathogens.

**Table 1**

Mean  $IL-1\beta$  and  $TNF-\alpha$  concentrations in spleen as a function of exposure conditions four days after the viral infection (S2). Concentrations are expressed in ngmg<sup>-1</sup> of protein for each contaminated conditions (G, R, V) and the control group (C) with standard errors for  $IL-1\beta$  ( $5 \leq n \leq 8$ ) and  $TNF-\alpha$  ( $8 \leq n \leq 10$ ). A significant difference from the control mean is indicated with a bold number and an “\*” ( $p < 0.05$ ).

Condition	C	G	R	V
$IL-1\beta$ (mean ± se)	1.70 ± 0.20	<b>0.65 ± 0.11*</b>	1.98 ± 0.57	2.63 ± 0.43
$TNF-\alpha$ (mean ± se)	10.85 ± 1.47	9.46 ± 1.17	9.24 ± 1.40	11.55 ± 1.36

## CRedit authorship contribution statement

**Jessy Le Du-Carrée:** Methodology, Investigation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Visualization. **Joëlle Cabon:** Investigation, Validation. **Lénaïg Louboutin:** Investigation, Validation. **Thierry Morin:** Validation, Writing – review & editing, Visualization, Supervision. **Morgane Danion:** Validation, Writing – review & editing, Visualization, Project administration.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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