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Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*

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Abstract

In the past few decades, glyphosate became the most used herbicide substance worldwide. As a result, the substance is ubiquitous in surface waters. Concerns have been raised about its ecotoxicological impact, but little is known about its generational toxicity. In this study, we investigate the impact of an environmentally relevant concentration of glyphosate and its co-formulants on an F2 generation issued from exposed generations F0 and F1. Trans, inter and multigenerational toxicity of 1 µg L^{-1} of the active substance was evaluated on early stages of development and juvenile rainbow trout (*Oncorhynchus mykiss*) using different molecular, biochemical, immuno-hematologic, and biometric parameters, behavior analysis, and a viral challenge. Reproductive parameters of generation F1 were not affected. However, developmental toxicity in generation F2 due to glyphosate alone or co-formulated was observed with head size changes (e.g. head surface up to +10%), and metabolic disruptions (e.g. 35% reduction in cytochrome-c-oxidase). Moreover, larvae exposed transgenerationally to Viaglif and intergenerationally to glyphosate and Roundup presented a reduced response to light, potentially indicating altered escape behavior. Overall methylation was, however, not altered and further experiments using gene-specific DNA metylation analyses are required. After several months, biochemical parameters measured in juvenile fish were no longer impacted, only intergenerational exposure to glyphosate drastically increased the susceptibility of rainbow trout to hematopoietic necrosis virus. This result might be due to a lower antibody response in exposed fish. In conclusion, our results show that generational exposure to glyphosate induces developmental toxicity and increases viral susceptibility. Co-formulants present in glyphosate-based herbicides can modulate the toxicity of the active substance. Further investigations are required to study the specific mechanisms of transmission but our results suggest that both non-genetic mechanisms and exposure during germinal stage could be involved.

Keywords: Glyphosate-based herbicide, Embryo-larval development, swimming behavior, viral challenge, oxidative stress, generational toxicity

1. Introduction

Human activities in modern societies involve particularly strong interactions with natural ecosystems [75]. In agriculture, production levels have increased significantly in the past few decades to respond to consumer demand, with the massive use of agrochemicals [101]. Among these chemical inputs, glyphosate is a widely used herbicide that has improved agricultural efficiency by controlling weed development [7]. Sprayed in the form of co-formulated products called glyphosate-based herbicides (GBHs), this active substance and its associated co-formulants have raised concern about their effects on aquatic ecosystems [38, 32, 39]. While glyphosate does not bio-accumulate in animal tissues [22, 25], it is semi-persistent in the aquatic environment for 7 to 14 days [38]. Its massive use therefore makes it ubiquitous in surface water, inducing almost continuous exposure with aquatic organisms.

Environmental risk assessments of glyphosate, like those carried out by the European Food Safety Authority (EFSA), classified the risk for aquatic organisms as low, considering both expected levels of this active substance in the environment and its toxicity parameters [25]. However, there is evidence that glyphosate alone possesses its own toxicity, often characterized by a non-monotonic dose-response curve (i.e. lower doses could induce greater effects) [102, 79, 95], and can distrupt certain physiological functions of aquatic organisms [1]. There is also considerable debate due to confusion between the toxicity of the active substance alone and that of GBHs, associated with a lack of clarity in certain research papers [74]. Some GBHs have been found to be more toxic than the pure active substance: this toxicity could be associated in part with the co-formulants or with their interactions with glyphosate [99].

In France, where a governmental plan has the objective to reduce its use from 50% for 2022, glyphosate was frequently detected in streams and river waters between 2007 and 2017 (ranging from 22.2 to 49.7%) [3] and a recent study reported mean concentration in surface water of $0.22\,\mu g\,L^{-1}$ of active substance [46]. At the European level, maximum predicted concentration in surface waters are comprised between 20 and $40\,\mu g\,L^{-1}$ [25]. Considering these concentration range, the majority of studies evaluating the effects of glyphosate and GBHs on fish were done with non-environmentally relevant concentrations. Although these concentrations are reported as sublethal [25], they could produce high toxic stress, inducing physiological disruptions that are not specific to the mode of action (MoA). Studies using doses at or near environmentally relevant concentrations have shown effects on acetylcholine esterase (AChE) [42], oxidative stress defenses [86, 5], parameters related to energy metabolism [71, 6, 42], and the immune system [26]. The changes reported at these levels of biological organization could be associated with observations at the level of the entire organism, such as early development disruptions [31, 111, 105], behavioral changes [113, 29, 37, 13, 105], or decreased resistance to pathogens [51]. However, no clear correlations between effects at different levels of organization have been highlighted, and the complex MoA of glyphosate alone or associated with co-formulants is still not understood [1].

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Direct exposure to a chemical is the most usual route of contamination that could impact the phenotype of an individual, and it is the most studied in the field of ecotoxicology. Nevertheless, in the last few decades, the transmission of toxicity through genetic and non-genetic mechanisms of heredity has become a particular source of concern [93]. It is now acknowledged that these mechanisms play a role in the adaptation of organisms to their changing environment [78]. While genetic mutations induced by the genotoxic properties of a chemical could mostly impact the natural population in the very long-term [9], non-genetic mechanisms such as epimutations are more likely to induce physiological changes in the short term [8]. Intergenerational and transgenerational exposures correspond to a generation F1 and at least a generation F2, originating from a contaminated F0 generation [98]. In the case of intergenerational exposure, the F1 generation is directly exposed at the stage of germinal cells in the parent organism. In transgenerational exposure in fish having external fecundation, the phenotype of the F2 generation is more likely affected by non-genetic inheritance, such as epigenetic mechanisms [44]. Finally, for contaminants that are ubiquitous in the environment such as glyphosate, multigenerational exposure is the most environmentally relevant mode of exposure because it considers fish originating from contaminated parents that are also directly contaminated [44]. While transgenerational toxicity has been demonstrated only in mammals, [55], inter and multigenerational toxicity in fish species has been identified by several authors [103, 95].

In previous studies, we observed that a chronic exposure of adult rainbow trout (F0) to an environmentally relevant dose of glyphosate of $1\,\mu g\,L^{-1}$ administered alone or associated with co-formulants, induced only occasional impact on immune response without major change in reproduction, metabolism nor oxidative response [57]. However, F1 fish born from this exposed F0 generation (i.e. intergenerationally exposed F1) showed behavioural changes and modified markers of energetic metabolism, depending of the presence and the nature of co-formulants [58]. To go further and bring evidence that non-genetic mechanisms of toxicity inheritance exist, we analyzed in this study the impact of glyphosate and GBHs multi, inter and transgenerational exposures on the F2 generation. We focused particularly on the early development of this F2 generation, with measurements of different biochemical parameters and the characterization of biometric and behavioral traits. Defense capacities against a viral infection were also evaluated in F2 juvenile fish and were interpreted in light of their energy metabolic status.

2. Materials and methods

2.1. Chemicals

Three chemical compounds were tested: glyphosate active substance (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6) and two GBHs: Roundup Innovert® (R; Agrilisa - for professional use) and Viaglif Jardin® (V; Agrilisa - for home gardens). The purity of G was 98%, and the concentrations of R and V were 360 and $420\,g\,L^{-1}$ of glyphosate, respectively. Formulation properties and concentrations of the two commercial products, R and V, were unknown. For each product, concentrated aqueous solutions ($4\,mg\,L^{-1}$ in distilled water) were prepared and stored under appropriate conditions (darkness, $4\,°C \pm$

2). A pre-dilution of pure glyphosate was done in 10 mL of pure methanol (concentration of solvent in concentrated solution was 10 mL L^{-1} so the final dose of methanol exposure was kept under 4 µL L^{-1} as recommend by Hutchinson et al. [45]).

2.2. 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). Euthanasia involved the 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.

A timeline describing the production of the three rainbow trout generations and the experimental design is presented in Figure 1.

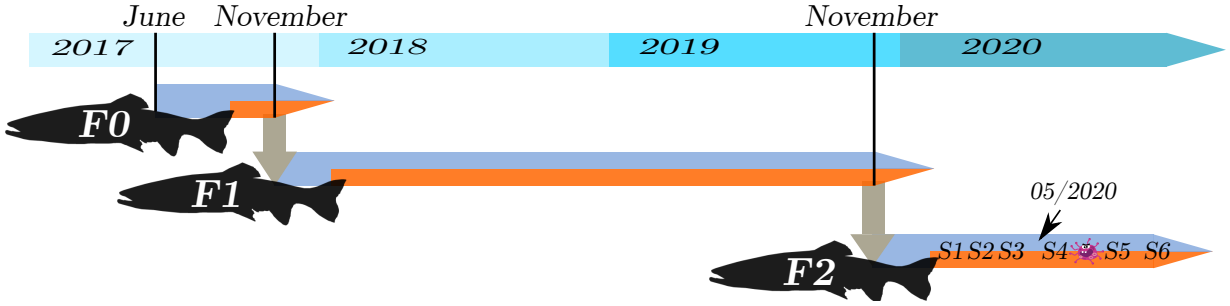


Figure 1: Timeline illustrating production of the different rainbow trout generations and the analyses carried out on water and fish samples for the F2 generation. For each generation (F0, F1, F2), full blue lines represent phases without chemical exposure. Colors on bars represent period with (orange) and without chemical contamination (blue; for details see Figure 2), and the control groups (in blue). Viral challenge is marked with a virus symbol. Sampling were done at larvae stage: S1 (350 degree-day, DD), S2 (541 DD), S3 (between 615 and 681 DD) and at junvenile stage: S4 (11 days before the *IHNv* challenge), S5 (4 days post-infection (dpi)), and S6 (42 dpi). Analysis of glyphosate and AMPA in water is indicated with an arrow, above which the period of analysis.

Fish were maintained in tanks of 40 L (juveniles) and 400 L (adult) containing river water filtered with sand filter (approximately 20 µm), 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 µS m^{-1} and a hardness of 12 °TH. Concentrations of ammoniac, 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. After the eyed stage period, embryos were placed in 8 tanks (40 L) positioned in a confined room. Temperature during embryonic development was maintained at 8 °C ± 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*.

The F0 and F1 generations were exposed daily for 10 days and 24 months, respectively, to a mean concentration of 123 ng L^{-1} of glyphosate using G, R, and V [57]. Unexposed control conditions (C) were included. F0 engendered the F1 generation (see [57] for more explanations on reproduction). The F2 generation was produced by 2 to 5 females and 2 to 8 males of the F1 generation from the conditions described in Figure 2. Conditions C/V and V/V were lost during the experiment due to material dysfunction, so 8 conditions only could give birth to the F1 generation.

Procedure of trout reproduction is described in the article of Le Du-Carrée et al. [57] and embryonic development until the eyed stage (see Bobe et al. [10]) was conducted in 300 L tanks containing two breeding boxes by tank and continuously renewed with river water (filtered with sand and cotton wool filters) with a flow rate of approximately 300 L h^{-1}.

2.3. Experimental design

2.3.1. Chemical exposure

Once the F2 larvae reached eyed stage, rainbow trout embryos were exposed to the 8 conditions presented in Figure 2. The name of the condition is described with three letters separated by a forward slash, i.e. the first column represents the exposure conditions of generation F0, the second and third represent the exposure conditions of generations F1 and the F2, respectively. Fish produced from non-contaminated F0 and F1 and not directly contaminated formed the control condition called C/C/C. Fish produced from non-contaminated F0 but directly contaminated in F1 were C/G/C, and C/R/C and are considered intergenerationally exposed fish. Fish only contaminated through F0 are considered transgenerationally contaminated, and were G/C/C, R/C/C, and V/C/C. Finally, multigenerational exposure designated fish that were contaminated continuously for three generations. They are represented by the conditions G/G/G and R/R/R. F1 and F2 chemical exposure was conducted with the same methodology as for F0 (details are available in Le Du-Carrée et al. [57]). In brief, every working day (generally 5 days a week), 10 mL of each of the respective concentrated chemical solutions were added to the experimental tanks, for which the arrival of water was stopped for one hour. Regulated water flow was set up after one hour of contact for the rest of the day at 13.5 L h^{-1}, resulting in gradual dilution of glyphosate. Theoretical glyphosate concentration kinetics were modeled using the equation 1 and the resulting curve is presented in Figure 4. The integrated mean daily theoretical concentration was approximately 123 ng L^{-1} (the area integrated is represented by a blue zone on the theoretical dilution curve in Figure 4).

$$C(t) = C_{initial} \times e^{-rate/V_{tank} \times time} \quad (1)$$

Water sampling was performed in March 2020 (after approximately three months of chemical contamination) to measure concentrations of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in water tanks. For each chemically contaminated and control tank, 150 mL water samples were taken using sterile plastic bottles and stored

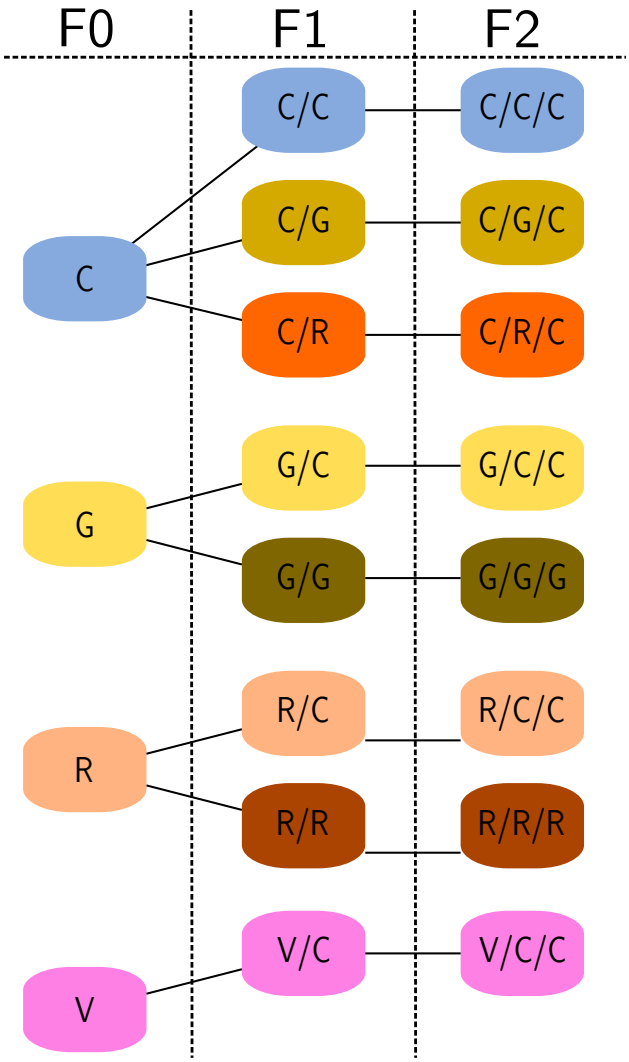


Figure 2: Origins and exposure status of the F2 generation after multi, inter and transgenerational exposure to glyphosate or GBHs. Conditions of chemical exposure are represented by the following letters: C, Control; G, Glyphosate; R, Roundup; V, Viaglif. F0: the letter corresponds to the condition of direct exposure of the genitors. F1: letter before the slash corresponds to the exposure condition of F0, while the letter after the slash corresponds to the condition of direct exposure of the F1 generation. F2: the letter before the first slash corresponds to the exposure condition of F0, while the letter between the slashes corresponds to the exposure condition of the F1 generation, and the letter after the second slash corresponds to the condition of direct exposure of generation F2.

at −4 °C. Quantification of glyphosate and AMPA was done within 72 hours after sampling using HPLC and fluorometric methods (Method ref. ANA-I10.MOA.69.B) by an external provider (Labocea, France).

2.3.2. Viral challenge

The virus used for viral challenge was the N61 strain (genotype E) of the infectious hematopoietic necrosis *IHN* virus (i.e. *IHNv*) isolated from diseased rainbow trout fry displaying typical signs of the disease. A 100 mL stock of the virus, isolated from diseases rainbow trout in 1989 and used as French reference strain, was produced at 14 °C on an *Epithelioma Papulosum Cyprini* (EPC) cell line [30] in homemade Eagle medium (Glasgow MEM in powder (Pan Biotech), with Tris-HCl 0.19 M and HCO3Na (pH 7.6) supplemented with 10% fetal bovine serum (Eurobio), 1X antibiotics (100 IU mL^{-1} penicillin G, 0.1 mg mL^{-1} streptomycin) and L-glutamine (HyClone). Once the cytopathic effect was complete, cell culture supernatant was centrifuged for 15 min at $2,000 \times g$ and stored at −80 °C. The infectious titer of the viral production, determined using the median tissue culture infectious dose ($TCID_{50}$) endpoint method in 96-microplate wells [49], was 4×10^7 $TCID_{50}$ mL^{-1}. After approximately 6 months of chemical exposure, 280 F2 fish for each chemical treatment were randomly distributed to four 10 L tanks with constant water renewal (i.e. 70 fish per replicate of a condition). Three of these tanks were infected with *IHNv*, and one was used as the uninfected control. Infection was done by placing fish in a reduced volume of 1 L highly oxygenated water with an infectious dose of 10^4 $TCID_{50}$ mL^{-1} for 3 hours. Non-infected EPC cell supernatant was used for uninfected control tanks.

For 6 weeks after *IHNv* infection, general behavior, the appearance of clinical signs (lethargy, darkening of the skin, exophthalmia), and mortality were recorded twice a day. Dead individuals were stored at −20 °C for viral examination.

2.4. Sampling date

Fertility was considered to be the proportion of eggs surviving at 5 days post-fertilization [17]. To perform this measurement, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes.

Larvae for biometric indices and malformation measurements were sampled at 350 DD (S1, see Figure 1 for graphical illustration) and stored in a 3% glutaraldehyde solution (described by Nikolakakis et al. [80]) at 4 DD until the analysis. Note that condition C/R/C was lost during storage and could not be analyzed.

At 541 DD (S2), invasive sampling was done on 20 larvae exposed to the different conditions. They were flash-frozen in liquid nitrogen and stored at −80 °C for future analyses.

Eleven days before infection (S4), 96 hours post-infection (96 hpi; S5), and 42 days post-infection (42 dpi; S6) to *IHNv*, invasive sampling was done on 20 juvenile rainbow trout aged approximately 6 months, with size and length (mean±se) of 5.69 g±5.69 g and 83.83 mm±0.75 mm, respectively. A blood sample was taken by withdrawing 10 µL of blood from the caudal vein with a lithium heparin hematocrit tube (Greiner ref. KG454244), and fish were euthanized. Then, at S4, gills were sampled, flash-frozen in liquid nitrogen and

stored at −80 °C for future analyses. Note that between S3 and S4, condition G/G/G was lost due to an incident during fish maintenance and could not be sampled.

2.5. Biomarkers analyzed

2.5.1. Biometric index measurements and malformation analysis

Biometric index measurements and malformation analysis were done on a picture of larvae taken with a binocular magnifier (Stemi 508) coupled to a camera (Canon DS126431) permitting 40X magnification. Body length (without tail fin), head surface, eye diameter, and yolk sac surface were analyzed using ToupView software, version 3.7 (Figure 3a) on a total of 30 larvae per condition. Frequencies of malformation were determined on larvae considering jaw malformations (Figure 3b), yolk sac edema (not observed), and spinal curvature (Figure 3c).

2.5.2. Swimming behavior analysis

The analysis was done during a time window of seven days with free-swimming larvae (i.e. from 615 to 681 DD, S3 sampling date in Figure 1) maintained at 11 °C throughout the experiment.

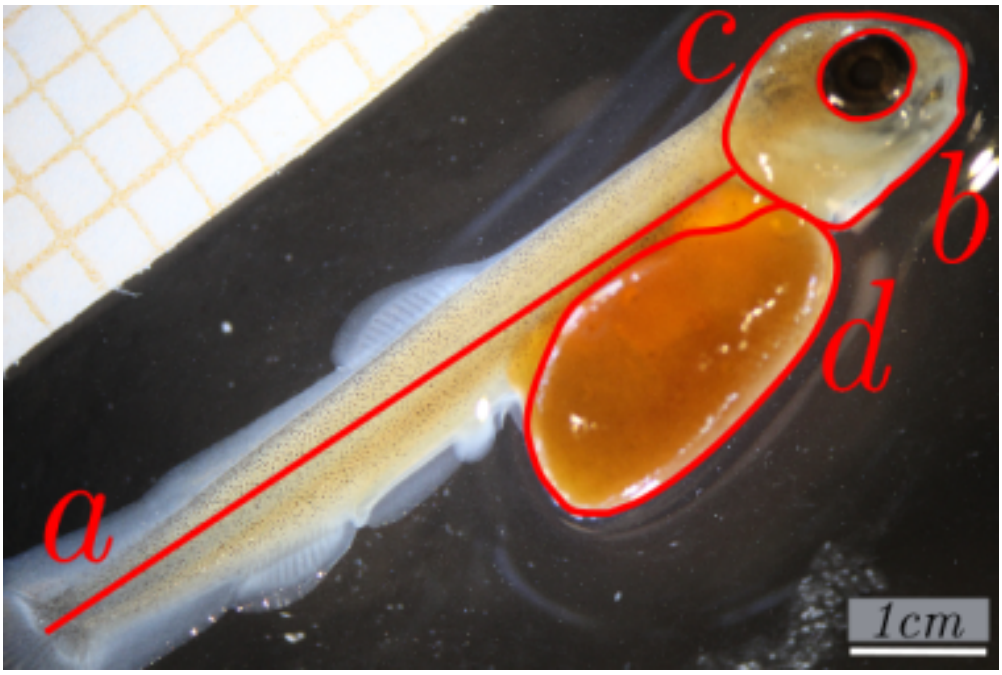
The protocol for the swimming photomotor assay was adapted from the study by Weeks Santos et al. [105] and is described in detail in Le Du et al. (submitted). in brief, a DanioVision system was used to record the distance and the speed travelled by larvae individually distributed in six-well cell culture plates during three phases at different light intensities (10 min of darkness, Dark 1 ; followed by 10 min of light, light 1 ; finally followed by 10 minutes of darkness, Dark 2).

2.5.3. Viral examination and immune parameters during the viral challenge

The presence and concentration of *IHNv* were checked individually from 3 dead fish collected at peak mortality, by replicate chemical condition. Extracted organs (kidneys, spleen, heart, and brain) were pooled and crushed using a mortar and pestle, diluted to 10^{-1} with Eagle medium, and centrifuged for 15 min at $2,000 \times g$ at 4 °C. The supernatant was then diluted to 10^{-8}, and the virus concentration was determined for each fish, as described in Section 2.3.2. At S5, red blood cell (RBC) and white blood cell (WBC) counts were performed on a Thoma cell hemocytometer using whole blood diluted to 1/200 in Giemsa solution [50]. At S6, detection and semi-quantification of anti-*IHNv* antibodies in the plasma of surviving fish were performed using a modified procedure following Jorgensen et al. [48], according to the repealed standard NF U-47-022 as described by Louboutin et al. [65].

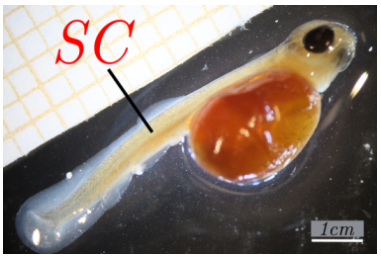
2.5.4. Methylation

Extraction of total DNA was performed using a DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. Global DNA methylation was then measured on 100 ng of total DNA extracted from whole fish larvae sampled at S2, using a MethylFlash Global DNA Methylation (5-methylCytosine or 5-mC) ELISA Easy Colorimetric Kit (Epigentek), following the manufacturer's instructions. Absorbance of the products were measured on a TECAN Spark 10M microplate spectrophotometer at 450 nm. Calculation of the



(a)

(b)



(c)

Figure 3: Type of malformations analyzed. Non-malformed control larvae (Sub-figure 3a) with indication of the different biometric indices measured: a. Body length, b. Head surface, c. Eye diameter and d. Yolk sac surface. Sub-figures 3b and 3c, represent directly-exposed larvae with malformations, respectively: jaw malformation (JM, from the G/C/C condition) and spinal curvature (SC, from the G/C/C condition).

percent methylated DNA for each sample was carried out by reporting the optical density values on the standard curve using the formula 2 (NC = negative control,A = absorbance, S = amount of total DNA in ng).

$$\text{5-mC\%} = \frac{A_{Sample} - A_{NC}}{Slope \times S} \times 100\% \quad (2)$$

2.5.5. Oxidative stress and metabolic parameters

Choline esterases (ChE), oxidative parameters, namely thiobarbituric acid reactive substances (TBARS), catalase (CAT), and glutathione peroxidase (GPx), and metabolic parameters, i.e. citrate synthase (CS); cytochrome c oxidase (CCO); lactate dehydrogenase (LDH); and glucose-6-phosphate dehydrogenase (G6PDH), were assayed in larvae (sampled at S2) and gills of juvenile (sampled at S4) following the same procedures described in a previous article [57].

2.6. Data processing and statistical analyses

Statistical analyses and data processing were performed with R software [88]. Figures were generated using the ggplot2 package [107]. Quantitative data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one-way ANOVA tests were used to compare means, followed by a post-hoc test of Dunnett [24]. In the case of normal and heteroscedastic data, modified one-way ANOVA tests were used to compare means [106], followed by a post-hoc test of Tamhane-Dunnett [82]. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn [23]. Differences between malformation rates were compared using a chi-squared test. Survival rates for the different chemical treatments were compared using the "survival" package [100]. A p-value of 0.05 was used as the threshold for statistical significance. A test of correlations between variables was carried out using the lmrob R function (R package robustbase; [108]).

3. Results

3.1. Glyphosate concentrations in exposure tank water

Validation of the experimental chemical contamination procedure for the exposure of F0 and F1 generation trout in 400 L tanks was performed in a previous experiment (see Le Du-Carrée et al. [57] for methodological details and results). During this experiment, the river water supplying the tanks did not present any detectable concentration of glyphosate. After one hour of exposure and just before water flow reopening, concentrations of glyphosate of 0.54 and 0.57 µg L^{-1} were quantified in tanks contaminated with glyphosate and Roundup, respectively (Figure 4). These concentrations were 46 and 43% below the theoretically expected value of 1 µg L^{-1}. One hour after water flow reopening, the concentration of glyphosate in the tanks contaminated with the active substance was close (2% variation) to the expected concentration of 0.71 µg L^{-1}, whereas it was 47% lower in the Roundup

contaminated tanks. Two hours after restarting water flow, glyphosate concentrations were 45% below expected values for both tanks (0.28 instead of 0.51 µg L^{-1} theoretically). AMPA, the main metabolite of glyphosate, was not detected in any of the samples including those artificially contaminated with the active substance or Roundup.

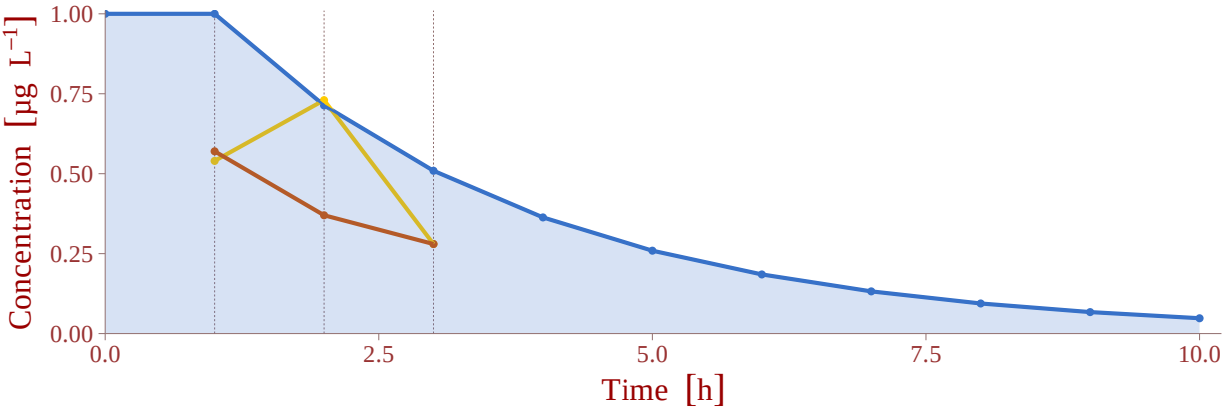


Figure 4: Mean concentrations of glyphosate in water tanks as a function of time (µg L^{-1}). Concentrations obtained by HPLC analysis were compared with theoretical concentrations (in blue, modeled using equation 1) at different kinetic time-points. Water was sampled after approximately three months of contamination for each condition (Glyphosate - G in yellow, Roundup - R in orange), just before, and 1 and 2 hours after water flow reopening.

3.2. Fertility and fecundity of the F1 generation

Relative fecundity of the F1 generation aged of two years varied between 1.79±0.61 and 3.05±0.40 eggs g^{-1}, regardless of the condition, with no detectable impact of the chemical contamination (Table 1).

Fertility was calculated for control and intergenerationally contaminated fish of the F2 generation. It was greater than 97% regardless of the condition considered (data not shown). No statistically significant difference was measured.

3.3. Biometric indices and malformations observed on the F2 generation

No differences between control and chemically contaminated F2 larvae sampled at S1 were observed for body length and yolk sac surface (Table 2). Different chemical conditions induced significant changes in other biometric indices, such as an increase in head surface ($p.value < 0.0001$, d.f.= 6 and $\chi^2 = 30.50$), eye surface ($p.value < 0.0001$, d.f.= 6 and $\chi^2 = 43.56$), and eye:head surface ratio ($p.value = 0.001$, d.f.= 6 and $\chi^2 = 26.97$). For head surface, a post-hoc test revealed significant differences for the G/C/C (+10%) and G/G/G (+9%) conditions, compared to the control ($p.value < 0.05$). For eye surface, a post-hoc test revealed a significant increase (+9%) for the multigenerationnally exposed G/G/G condition ($p.value < 0.05$). For eye:head surface ratio, a post-hoc test revealed a significant reduction for C/G/C (-5%) and R/R/R (-9%) compared to the control ($p.value < 0.05$).

Table 1: Relative fecundity of the F1 generation (expressed in eggs g^{-1}; mean ± standard error, se) as a function of the chemical exposure conditions ($2 \leq n \leq 5$; see Figure 2).

Mode of exposure	Condition	**Relative fecundity**	
		mean	se
Control	C/C	2.17	0.44
Trans.	G/C	2.85	0.28
	R/C	2.10	0.33
	V/C	3.05	0.40
Direct	C/G	2.27	0.38
	C/R	1.79	0.61
Multi.	G/G	2.14	0.86
	R/R	2.53	0.44

Spinal curvatures and jaw malformations were detected in F2 larvae (Figure 5). Low frequencies of jaw malformations were observed for all conditions, with proportions ranging from 0 to 7%. Spinal curvature was the most frequent malformation detected, with frequencies ranging from 0 to 11%. No yolk sac edema was observed. Chemical treatments did not induce statistically significant induction of malformations compared to the non-exposed condition (i.e. the control).

3.4. Metabolic activity

Chemical contamination induced changes in certain enzymatic levels in F2 larvae sampled at S2 (Table 3). While no changes were observed for AChE, LDH, or TBARS regardless of the chemical condition considered, statistically significant reductions in enzymatic activities were detected for CAT, CCO, and CS. CAT activity was affected by chemical exposures ($p.value = 0.003$, d.f.= 7 and $f = 3.25$), with a significant reduction of 13, 18, 15, and 23% found by a post-hoc test for the G/C/C, G/G/G, R/C/C and V/C/C conditions, respectively, compared to the control ($p < 0.05$). CCO and CS activities were also affected by chemical exposure ($p.value < 0.0001$, d.f.= 7 and $f = 12.30$; $p.value = 0.04$, d.f.= 7 and $f = 2.14$, respectively), with reductions for G/C/C and V/C/C comprised between 12 and 33%, compared to the control ($p < 0.05$). A reduction of 35% in CCO activity was also observed between the control and G/G/G ($p < 0.05$). The ratio between CS and CCO activities (CS:CCO ratio), which presented mean values ranging between 138.32 ± 4.38 and 206.76 ± 15.41, was affected by chemical contamination ($p.value < 0.0001$, d.f.= 7 and $\chi^2 = 53.60$). Increases of 33 and 31% were observed for G/C/C and G/G/G compared to the control, respectively ($p < 0.05$). The LDH:CS ratio, comprised between 3683.89 ± 84.00 and 4656.21 ± 127.94, was also altered by the chemical treatments ($p.value < 0.0001$, d.f.= 7 and $f = 11.39$). Conditions G/C/C, C/G/C, and V/V/V showed increased values of 19, 24, and 14% compared to the control, respectively ($p < 0.05$).

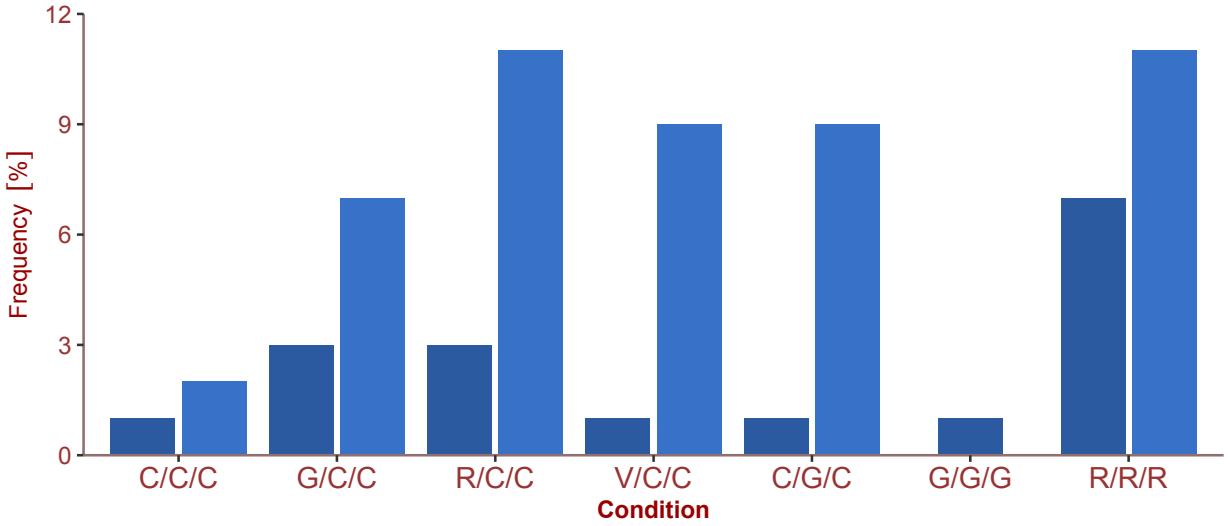


Figure 5: Malformation frequencies measured in F2 larvae at 350 DD (S1) according to the different chemical conditions ($89 \leq n \leq 113$; see Figure 2). Bars represent jaw malformation frequencies (dark blue) and spinal curvature frequencies (pale blue). The analyses were done on a total of 89-113 larvae per condition.

Table 2: Mean biometric indices measured at 350 DD (S1) in F2 larvae exposed directly or through their parents to glyphosate or GBHs (see Figure 2 for details on chemical exposure conditions). Standard errors are given in parentheses under each respective mean ($n = 30$). Lengths are expressed in mm, surfaces in mm^2 and ratios in %. Numbers in bold with an asterisk indicate conditions significantly different ($p < 0.05$) from the control.)

Parameter	**Mode of exposure**						
	Control	**Transgenerational**			**Inter.**	**Multigenerational**	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	G/G/G	R/R/R
Body length	14.9 (0.121)	15.35 (0.148)	15.01 (0.113)	15.15 (0.117)	15.12 (0.091)	15.42 (0.09)	14.36 (0.186)
Head surface	6.25 (0.089)	**6.89*** (0.196)	6.32 (0.138)	6.39 (0.129)	6.47 (0.109)	**6.82*** (0.114)	5.85 (0.179)
Eye surface	1.17 (0.023)	1.34 (0.097)	1.21 (0.022)	1.2 (0.025)	1.15 (0.018)	**1.28*** (0.03)	1.01 (0.051)
Eye:head surface ratio	18.78 (0.29)	19.11 (0.71)	19.18 (0.27)	18.8 (0.25)	**17.89*** (0.2)	18.71 (0.4)	17.04 (0.67)

At S4, chemical contamination did not induce changes in LDH and GPx activities nor in CS:CCO and LDH:CS ratios in rainbow trout gills (See Table B.6 in Appendix). However, changes were observed in CCO and CS activities ($p.value = 0.002$, d.f.= 6 and $f = 3.87$ and $p.value = 0.003$, d.f.= 6 and $\chi^2 = 19.94$), enzymes involved in aerobic metabolism. Mean CCO activity ranged between 0.16 ± 0.0089 and $0.26 \pm 0.011\,\mathrm{IU\,mg^{-1}}$, while CS activities ranged between 0.32 ± 0.0098 and $0.36 \pm 0.0082\,\mathrm{IU\,mg^{-1}}$ according to the considered treatment. A post-hoc test revealed that CCO and CS activities were 24% higher in the C/G/C condition compared to the control ($p < 0.05$) (See Table B.6 in Appendix).

Table 3: Mean specific activities and TBARS levels measured in whole F2 larvae at 541 DD (S2) for the different chemical conditions (see Figure 2). Specific activities are expressed in IU mg^{-1} of protein and MDA concentrations in nmol mg^{-1} of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). Values in bold with an asterisk are significantly different ($p < 0.05$) from the control condition.

Parameter	**Mode of exposure**							
	Control	**Transgenerational**			**Intergenerational**		**Multigenerational**	
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	G/G/G	R/R/R
AChE	0.31 (0.0094)	0.29 (0.007)	0.31 (0.0079)	0.28 (0.0099)	0.29 (0.0117)	0.31 (0.0105)	0.3 (0.011)	0.35 (0.0145)
CAT	7.46 (0.21)	**6.3*** (0.27)	**6.32*** (0.29)	**5.78*** (0.25)	6.46 (0.27)	6.31 (0.26)	**6.14*** (0.33)	6.96 (0.38)
CCO	0.25 (0.0173)	**0.16*** (0.0089)	0.23 (0.0076)	**0.2*** (0.0084)	0.23 (0.0113)	0.22 (0.0089)	**0.16*** (0.007)	0.26 (0.0109)
CS	0.36 (0.0082)	**0.32*** (0.0098)	0.34 (0.0092)	**0.32*** (0.0117)	0.32 (0.0128)	0.34 (0.0123)	0.32 (0.0116)	0.35 (0.013)
CS:CCO	1.56 (0.111)	**2.07*** (0.154)	1.54 (0.047)	1.59 (0.056)	1.47 (0.066)	1.6 (0.066)	**2.03*** (0.051)	1.38 (0.044)
LDH	13.4 (0.5)	14.05 (0.4)	14.41 (0.52)	13.5 (0.58)	14.89 (0.59)	12.54 (0.47)	12.07 (0.53)	13.03 (0.65)
LDH:CS	37.44 (1.53)	**44.65*** (1.24)	42.7 (1.34)	**42.68*** (1)	**46.56*** (1.28)	36.84 (0.84)	37.53 (0.95)	37.33 (0.87)
TBARS	0.92 (0.284)	0.57 (0.114)	1.18 (0.198)	0.54 (0.064)	1.07 (0.104)	0.97 (0.095)	0.65 (0.077)	0.82 (0.095)

3.5. Global methylation in whole F2 larvae

The proportion of 5-methyl Cytosine (5-mC) measured in total DNA of whole larvae, comprised between 2.68 ± 0.26 and 3.42 ± 0.21 5mc/total DNA, was similar among the different chemical exposure conditions (Figure 6).

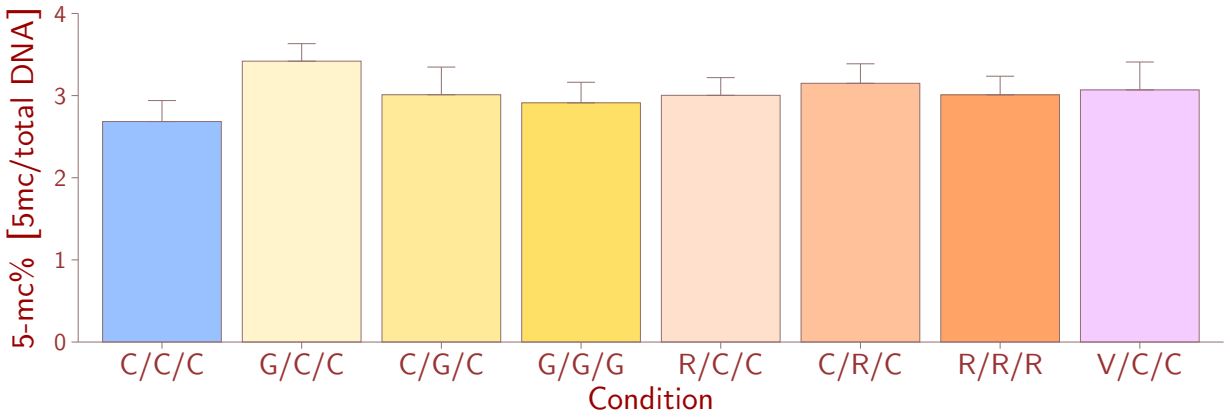


Figure 6: Mean global DNA methylation expressed in 5-methyl Cytosine % (5mc/total DNA) measured in whole larvae for each chemical condition ($8 \leq n \leq 9$, see Figure 2).

3.6. Swimming behavior

Distances traveled by larvae in darkness were comprised between 4.18 ± 0.64 m and 6.89 ± 1.11 m and between 7.97 ± 0.69 m and 10.16 ± 0.89 m (mean±se) during the first and second periods, respectively (Figure 7). A drastic speed reduction was observed under light exposure, with distances traveled comprised between 1.87 ± 0.24 m and 2.65 ± 0.40 m. Chemical exposure did not induce statistically significant changes in the traveled distance for the different light intensity periods considered. However, a peak of swimming activity was observed during the minute following opening of the light (blue area of the Figure 7, where larvae from the control condition presented the highest speed. The figure 8 represent the speed of larvae during the minute following the opening of the light. A comparison of this values for the different chemical exposure conditions revealed a statistically significant effect of chemical exposure on the response to light ($p.value = 0,004$, d.f.$= 7$ and $\chi^2 = 20,35$). A post-hoc test showed that control speed was 47, 40 and 42% higher than C/G/C, C/R/C, and V/C/C, respectively ($p < 0.05$).

3.7. Mortality induced by the viral challenge

Mortality was observed for all conditions following the viral challenge with *IHNv* (Figure 9). The lowest cumulative mortality rate was obtained for the non-chemically exposed larvae (i.e. $39.1 \pm 5.6\%$, mean $\pm$ se, n $= 3$), while values ranging from $46.0 \pm 1.6\%$ to $83.0 \pm 5.7\%$ were obtained for chemically exposed fish, with a significant difference between groups

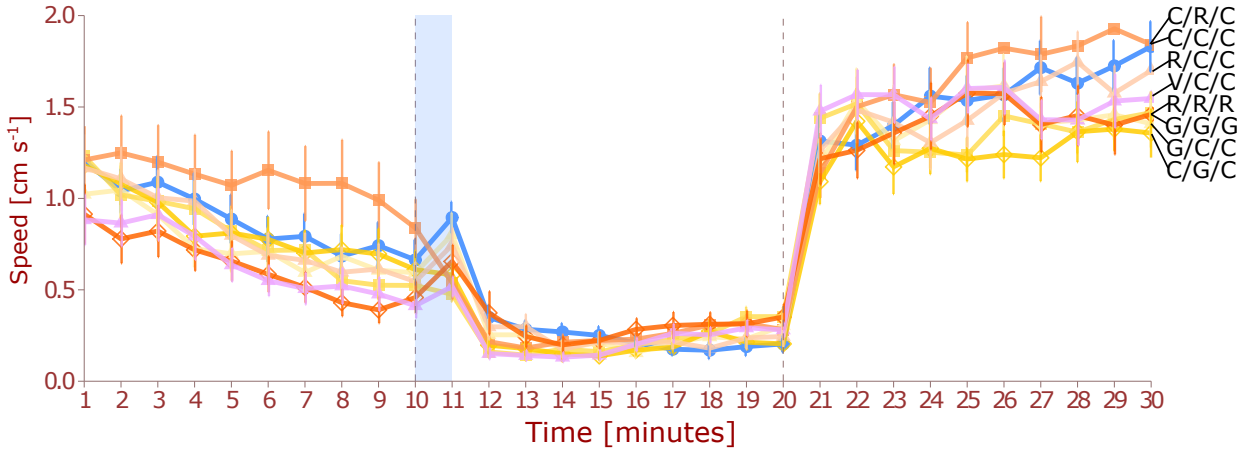


Figure 7: Mean speed of the F2 larvae for the different phases of light intensities according to the exposure condition (see Figure 2). Mean speeds, expressed in $\mathrm{cm\,s^{-1}}$, were analyzed at S3. Standard errors are given at the top of each bar ($33 \leq n \leq 46$). Colors indicate exposure conditions: G (in yellow), R (in orange), V (in pink), and control (in blue). For chemically contaminated groups, solid circle, solid square, and non-solid diamond represent trans, inter, and multigenerational exposure, respectively. The first and second dashed vertical bars represent the opening and extinguishing of the light, respectively. The blue rectangle indicates the minute of peak swimming activity at the start of the light phase, during which the larval speeds under the different conditions are compared in Figure 8

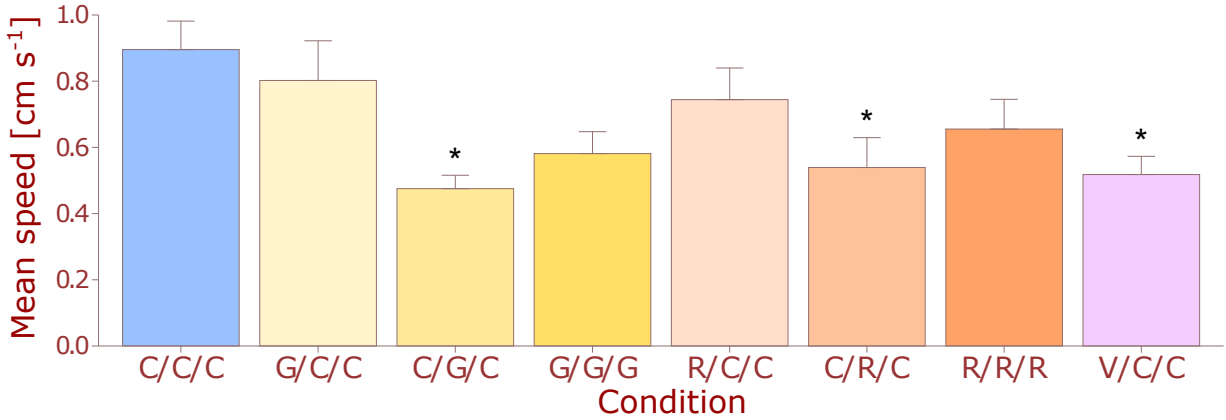


Figure 8: Mean speed ($\mathrm{cm\,s^{-1}}$) of F2 larvae during the minute after light opening as a function of exposure conditions. Standard errors are given at the top of each bar ($33 \leq n \leq 46$). Significant differences from the control means are indicated with "*" ($p < 0.05$).

($p.value < 0,0001$, d.f.= 6 and $\chi^2 = 234$). A post-hoc test revealed a significant difference ($p.value < 0,05$) in mortality between the C/G/C, R/R/R, and C/R/C conditions, which presented restricted mean survival times (RMSTs) of 14.6 ± 2.6, 23.0 ± 3.9, and 25.3 ± 2.5 dpi (mean ± se, n = 3), compared to 32.9 ± 1.1, 33.8 ± 0.5, 33.8 ± 1.1, and 36.6 ± 2.1 dpi for the control, V/C/C, R/C/C and G/C/C conditions, respectively.

Maximum daily mortality (i.e. the mortality peak) was observed 6 to 10 dpi for all conditions (data not shown). On the three pools of dead fish analyzed at the mortality peak, *IHNv* was detected in 33.3 to 100% of the samples, depending on the exposure condition (Table 4). The mean viral titer of positive pooled fish was between $2.38 \times 10^9 \pm 1.97 \times 10^9$ and $4.88 \times 10^3 \pm 1.76 \times 10^3$ for the different conditions of chemical contamination, and was not correlated with the cumulative mortality observed.

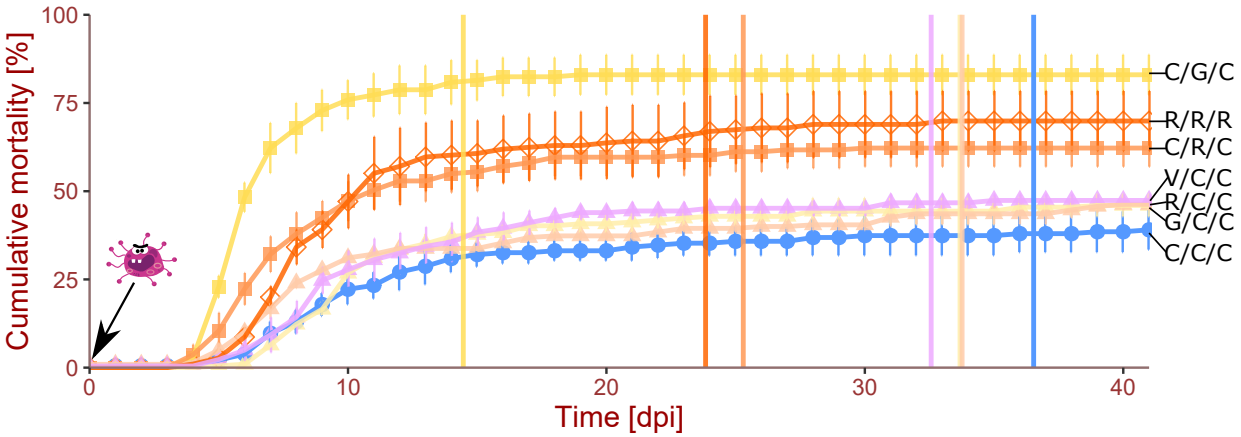


Figure 9: Cumulative mortality in chemically exposed fish (see Figure 2 for details on chemical exposure conditions) infected with *IHNv*. Infection time point is marked with a virus symbol. Data are expressed as a function of time in days post-infection (dpi). Colors represent chemical contaminants: G (in yellow), R (in orange), V (in pink) and control (in blue). For chemically contaminated groups, solid circle, solid square, and non-solid diamond represent trans, inter, and multigenerational exposures, respectively. Condition names have been indicated at the end of each respective curve to facilitate reading. Error bars represent standard errors, vertical bars the restricted mean survival time (RMST).

3.8. Immuno-hematologic parameters

After 96 h of viral infection (S4), no effects of chemical contamination were detected in the RBCCs or WBCCs of the F2 juvenile rainbow trout (see Table A.10 in Appendix). RBCCs ranged between 0.32 ± 0.031 and 0.56 ± 0.11 TL, while WBCCs ranged between 15.69 ± 1.55 and 25.5 ± 3.97 GL.

At 42 dpi (S6), similar proportions of *IHNv* seropositive fish, ranging between 10/15 and 14/15 fish, were detected in the serum of the survivor fish for all exposure conditions (Table 5). However, the control and transgenerationally exposed fish presented higher proportions of highly seropositive fish (comprised between 60 and 85%), compared with fish intergenerationally exposed to glyphosate and Roundup and multigenerationally exposed to Roundup (comprised between 36 and and 55%). No statistically significant differences in proportions

Table 4: Proportion of *IHNv*-positive pools of fish per condition (see Figure 2) and mean viral titers at the mortality peak. Viral titers are expressed in 50% tissue culture infectious dose per mL ($TCID_{50}\,mL^{-1}$) as means ± standard error (n = 3).

Condition	**Parameters**		
	Proportion of *IHNv*-positive fish	Mean titer ($TCID_{50}\,mL^{-1}$)	se
C/C/C	3/3	2.38×10^9	1.14×10^9
G/C/C	1/3	6.32×10^3	-
R/C/C	2/3	1.03×10^6	6.85×10^5
V/C/C	2/3	4.88×10^3	8.31×10^2
C/G/C	2/3	3.26×10^5	2.16×10^5
C/R/C	3/3	7.40×10^7	3.65×10^7
R/R/R	3/3	8.84×10^4	3.38×10^4

of highly seropositive fish were found, but the p-value was just above the significance threshold ($p.value = 0.10$, d.f.= 6 and $\chi^2 = 10.51$). Nevertheless, the highly positive individuals presented mean anti-*IHNv* antibody titers that were not significantly affected by chemical contamination.

Table 5: Proportions of *IHNv*-seropositive and highly seropositive survivors per condition (see Figure 2) and mean anti-*IHNv* antibody titers (mean ± standard error, n = 15) at 60 dpi (S5). High seropositivity is defined as specific antibody titers greater than ≥ 640. The proportion of highly seropositive fish per condition corresponds to the ratio between the number of fish with a titer greater than ≥ 640 and the total number of seropositive fish. Mean antibody titers were calculated for highly seropositive fish only.

Condition	**Parameters**			
	Seropositivity	High seropositivity frequency (%)	Mean anti-*IHNv* antibody titer	se
C/C/C	11/15	73	1360	282
G/C/C	10/15	60	1280	286
R/C/C	12/15	83	1280	165
V/C/C	13/15	85	2153	369
C/G/C	13/15	54	3474	813
C/R/C	11/15	55	2560	859
R/R/R	14/15	36	1280	351

4. Discussion

Data on the generational toxicity of glyphosate are still rare, and few authors have reported transmission of deleterious effects from generation to generation in fish [103, 95] and mammals [55]. The complex experimental design of our study made it possible to examine the impact of both pure glyphosate and two GBHs (i.e. co-formulated glyphosate) on an F2 generation of rainbow trout (*Oncorhynchus mykiss*) chronically exposed to the contaminants at 1 µg L^{-1} of active substance either directly or through their parents and/or grandparents. This long-term experimental work provided the opportunity to investigate the effect at low dose of trans and intergenerational modes of toxicity transmission, but also the potential cumulative effects of multigenerational exposure, which best reflects the environmental reality.

The exposure procedure was validated by glyphosate detection at several timepoints of chemical dilution. Overall results revealed that glyphosate concentrations were approximately 50% below expected values after one-hour of contact and before water flow reopening. These results could be explained by poor homogenization of the chemical solution in the water tanks containing the fish. Concentrations found in tanks exposed to pure glyphosate two hours after water flow reopening matched well with expected values and appear to confirm this hypothesis. Nevertheless, values obtained after this first hour of exposure in a closed circuit seemed to fluctuate over the following days, as suggested by other assays carried out for the F0 and F1 generations at the same kinetic timepoints [57]. Additionally, mean concentrations could be closer to the theoretical values than those observed here.

Glyphosate alone or associated with co-formulants disrupt certain physiological processes linked to reproduction [4, 63, 97, 112, 92, 14, 47, 83]. While these effects are generally de-

tected at high, non-environmentally relevant concentrations, changes in 17β-estradiol levels, increased ovary diameter and disrupted ultrastructure, and induced over-expression of a gene involved in endocrine control of ovarian maturation have also been reported at lower concentrations in male delta smelt (*Hypomesus transpacificus*) [47] and female zebrafish (*Danio rerio*) [4, 19]. The lower dose of 1 µg L^{-1} used in the present study did not affect the fecundity and the fertility of the F1 generation submitted to direct, inter and multigenerational exposure to the active substance or the two GBHs. This is in phase with Smith et al. [95] who showed no evident effects of chronic exposure to 0.5 mg L^{-1} of pure and co-formulated glyphosate on the fecundity and fertility of Japanese medaka fish (*Oryzias latipes*) but only some changes in the expression of certain genes involved in spermatogenesis. At the same concentration, no impact was detected on female zebrafish, but at a dose 20 times higher, decreased fecundity without an effect on fertility was observed [103]. Interestingly, in this study, the highest dose tested increased the mortality rate in the F1 generation, but only when this generation was also directly exposed; no effects were found for intergenerational exposure.

Certain studies examining the effect of glyphosate on fish [95] or mammal species [55] have shown that while no effects were observed in the F0 generation, toxicity could be detected in later generations. In our experiment, the malformation occurrence rates in the F2 generation were not increased with statistical significance, regardless of the chemical exposure condition considered. However, spinal curvature frequencies tended to be higher (between +7 and +9% compared to controls) in all transgenerationally contaminated conditions (i.e. G/C/C, R/V/V, and V/C/C), but also in fish exposed intergenerationally to glyphosate (i.e. C/G/C) and multigenerationally to Roundup (i.e. R/R/R). Changes in biometric indices were also detected, particularly for the head surface and for the eye:head surface ratio, which were disrupted in several trans, inter and multigenerational conditions. In common carp (*Cyprinus carpio*) and zebrafish exposed during early development, no effects were observed on zebrafish, but an increase of the malformation rate was detected in common carp with low doses of pure glyphosate (i.e. 5 µg L^{-1}) [31]. During embryo-larval development of zebrafish, only doses of active substance greater than 100 mg L^{-1} induced decreases in body length and head and eye area [113]. In this species, increased biometrics and developmental malformation rates were shown only for concentrations above 8.5 mg L^{-1} [56]. No change in the malformation rate was reported in rainbow trout exposed for 3 weeks at the eyed stage to different doses of glyphosate (between 0.1 and 1 mg L^{-1}), but a decrease in the head:total length ratio of larvae was reported by Weeks Santos et al. [105]. During the early development of Japanese medaka fish, a 15-day exposure to 0.5 mg L^{-1} of both pure and co-formulated glyphosate induced embryo-larval malformations (e.g. spinal curvature, yolk sac edema) in the F1 generation, with greater intergenerational effects with pure glyphosate than with a GBH [95]. Since the sample size in our study may have been too low to detect a significant increase in malformation rates, further investigations are needed to show whether generational glyphosate exposure is able to induce skeletal abnormalities, at environmental concentrations. Moreover, our experiment appears to indicate an effect on head development in the case of trans, inter or multigenerational exposure, depending on the presence and on the nature of co-formulants. Despite statistical significance, the

changes observed were subtle (maximum 10% change compared to controls), and further analyses confirming these data are required. In addition, an evaluation of the impact of these developmental defects could reveal the biological significance of our results.

Alongside effects on embryo-larval morphology, behavioural changes have been detected after exposure of fish to pure and formulated glyphosate [113, 105]. Our analysis of swimming behavior indicates that while no effect of chemical contamination was observed on the speed (or distance traveled) of larvae during the different light phases, the response to light stimulation was disrupted. The light flash induced a considerable stress response characterized by a brief speed increase before larvae considerably decreased their exploratory behavior. This brief momentary speed increase was reduced in all exposure conditions, but particularly in intergenerational exposure to glyphosate and Roundup, and transgenerational exposure to Viaglif. This finding provides evidence that exposure of previous generations of fish could impact escape behaviour, and thereby the ability of new generations to survive in the natural environment (e.g. by altering the ability to avoid predators). Similar behaviour or conversely increased startle response were already reported using similar protocols in rainbow trout larvae exposed chronically or adult zebrafish after acute exposure [105, 29], with generally a non-monotonic response. This heterogeneity of response could be related with the life stages considered and the condition of exposure (mode, duration, and concentration of chemical exposure). Among the mechanisms associated with these disturbances, an increased anxiety was detected in adult zebrafish exposed to 0.3 and 3 $\mu g\,L^{-1}$ of glyphosate, with a significant increase in dopamine and serotonin levels as well as in the dihydroxyphenylacetic acid/dopamine and homovanillic acid/dopamine turnover ratios in the anterior brain [28, 83], and a deregulation of gene pathways directly involved in neuronal physiology and synaptic transmission (glutamate receptor, GABA receptor, cation channels ...) [33] were recently suggested.

The effects reported for the active substance in our study might not be mediated only by epigenetic mechanisms because intergenerational exposure induced a stronger effect than transgenerational exposure, demonstrating that direct chemical contact of the germinal cells could also be involved in the toxicity. Co-formulants, particularly those contained in Viaglif, induced a more accentuated transgenerational effect through potential epigenetic-mediated toxicity, due to their own toxicity or to their interactions with glyphosate.

Epigenetic mutations, including DNA methylation, have appeared in recent decades as a mechanism of non-genetic inheritance that can affect the phenotype of new generations, conferring physiological adaptations to cope with changes in the natural environment [78, 8]. Global DNA methylation results for our F2 larvae indicated only a slight trend (i.e. non-statistically significant compared to controls), with an increase for all chemically exposed fish (between +8% and +27% more than the control group). Changes in DNA methylation have been correlated with fish responses to certain stresses [60, 27], like environmental contaminants [110, 34]. Gene-specific methylation could have a significant impact on the phenotype of individuals, even though global methylation is not affected [21, 11]. When differential DNA methylation patterns are observed, they could be inherited from parents [35] but could also arise from methylation reprogramming during larval development due to the impact of exposure [21]. Chronic exposure of Japanese medaka fish during early life

stages to 0.5 mg L^{-1} of glyphosate or a GBH revealed changes in epigenetic-related genes in adult male gametes, and in the intergenerationally exposed F1 larvae [95]. However, the authors did not demonstrate a direct link between epigenetic changes and toxic effects observed in larvae, nor demonstrate whether these changes could persist in adulthood.

The MoA of glyphosate has not yet been clearly identified but generations of oxidative damages have been suggested to be involved in the induction of toxic effects by the active substance associated or not with its co-formulants [1, 83]. Thus activated markers of disruption of pro-oxidant anti-oxidant balance could indicate toxic effect of glyphosate at the cellular level. In the case of direct exposure, glyphosate and particularly GBHs generated oxidative stress in fish [5, 43, 42, 73, 92, 76, 94, 77, 12]. Oxidative stress is generally detected at high, non-environmentally relevant doses of the active substance [86][94] but a significant increase in the catalase and superoxide dismutase activities, coupled with a concomitant decrease of glutathione stores, was recently reported in the brain of adult zebrafish exposed two weeks with 0.3 and 3 µg L^{-1} of glyphosate [28]. Our results on entire larvae confirmed a reduced catalase activity, enzyme involved in the first line of defense against reactive oxygen species (ROS) [68], in larvae transgenerationally exposed to glyphosate, Roundup, and Viaglif, but also multigenerationally exposed to glyphosate. This phenomenon might be associated with epigenetic inherited regulations. Furthermore, the absence of increased TBARS levels, a commonly used biomarker of lipid peroxidation [68, 28], could suggest that no excess ROS levels were generated by direct or generational contamination. The decrease in catalase activity could be an effect inherited from the contaminated F0 generation or a non-specific effect due to general modulation of metabolism. Since the TBARS assay may not be sensitive enough to reveal subtle oxidative damage [72, 2] and was targeted on entire larvae and not on a specific tissue, further studies on the generation of oxidative stress associated with generational exposure might be useful.

. A well-documented effect of glyphosate exposure is its impact on energetic metabolism [52]. Detecting metabolic modification at the enzymatic levels could reveal a specific effect of glyphosate or its co-formulatants at cellular or infra-cellular levels but could also be associated to more global physiological perturbations. In fact, metabolic trade-off are commonly observed during stress events [96]. Chronic exposure of fish to glyphosate alone or co-formulated glyphosate induced changes in the expression of genes related to energetic metabolism, even at a concentration as low as 1 µg L^{-1} [71, 104]. Fish exposures to GBHs were also reported to disrupt different biochemical parameters at concentrations ranging from 26.5 µg L^{-1} to 298 mg L^{-1} [6, 42, 91, 20, 67, 40, 64, 41, 36, 15, 61, 59]. Our results indicate that aerobic metabolism, represented by the two key enzymes CCO and CS [81], was reduced in the case of transgenerational exposure to glyphosate and Viaglif, but also multigenerational exposure to glyphosate. While no change in the activity of LDH, an enzyme recognized as a good marker of anaerobic metabolism [18], was observed, activation of anaerobic metabolism versus aerobic metabolism was revealed by a higher LDH:CS ratio in larvae exposed transgenerationally to glyphosate and Viaglif, and intergenerationally to glyphosate [87]. The higher CS:CCO ratio detected could indicate mitochondrial dysfunction in F2 larvae exposed through the F0 generation to glyphosate (variation observed in trans-

generational and multigenerational modes of exposure). The increase of this ratio could be hypothetically associated with higher anabolic demand of mitochondria (i.e. non-essential amino acids, nucleotides, and fatty acid biosynthesis) [109, 16]. Several authors have shown that GBHs were able to affect mitochondrial function in isolated mitochondria [84], cells [66], and fish [85, 63, 19]. More specifically, inhibition of CCO activity and mitochondrial impairments in the brain of zebrafish were reported after chronic direct exposure to 65 µg L^{-1} of a GBH [85]. Therefore, the increase in the CS:CCO ratio we observed could also indicate that CCO was more strongly affected than CS by epigenetic regulations inherited from the previous generations of rainbow trout. Analyses of metabolic parameters in our juvenile trout revealed that changes observed during early development were no longer found after several months of life. The only persistent effect was an impact of intergenerational exposure to glyphosate on the two enzymes of aerobic metabolism. No impact was observed on GPx, another enzyme involved in defense against ROS, suggesting that no excess oxidative stress was produced by chemical contamination [68].

. Our fish were submitted to a viral challenge, a useful tool used to explore the potential toxicity of the different modes of chemical exposure on the immune system [89]. The impact of glyphosate exposure on the ability to survive viral infections has not been well studied. Acute exposure of juvenile silver catfish to a GBH at the concentration of 730 µg L^{-1} induced changes in immune cell parameters and a higher susceptibility to a bacterial challenge with *Aeromonas hydrophila* [54]. In our study, whereas transgenerational exposure did not alter survival following *IHNv* infection, significant impacts of intergenerational exposure to glyphosate and Roundup and multigenerational exposure to Roundup were observed. Therefore, toxicity transmission resulting in greater susceptibility of juvenile rainbow trout to virus infection could be associated with an epigenetic variation transmitted from contaminated parents to their descendants, but lost from an F0 to an F2 generation. More likely, the intergenerational effect could result from direct contact at the germinal stage with the contaminants. As the effect of contaminants on energetic metabolism cannot be correlated with the higher susceptibility of exposed rainbow trout, other physiological impacts, such as immune toxicity must be involved. However, no differences in the blood cell counts after 96 h of exposure were shown, when comparing the different chemical treatments. The only immuno-hematologic parameter analyzed that could explain this toxic effect was the proportion of highly seropositive fish. In fact, all the intergenerationally exposed fish presented a lower proportion of fish that had developed a strong anti-*IHNv* antibody response. As a result, the higher mortality related to viral infection observed for intergenerationally exposed fish could be associated with their inability to induce an effective antibody response. GBH exposure has been reported to modulate expression of immune-related genes [104, 62, 69, 90, 114] in fish, but also to disrupt the immune system [26, 54, 53, 62, 69, 70]; however, concentrations tested were often higher than ours. At the same concentration we used (i.e. 1 µg L^{-1}), deregulation of the immune gene *Fucolectin-1* was observed in European flounder (*Platichthys flesus*) after 30 days of exposure [71]. Therefore, further studies using a similar experimental design with generational exposure followed by a viral challenge, and integrating more specific immune biomarkers, are needed to investigate more in-depth the

mechanisms involved in this higher susceptibility to viral infections.

5. Conclusions

Our complex experimental design was an efficient approach to investigate generational effects on rainbow trout exposed to an environmentally relevant concentration of both pure glyphosate and two GBHs. Although no impact was observed on certain reproductive parameters of the F1 generation, the early development of the F2 generation was affected by chemical exposure of previous generations, with effects observed on metabolism, biometrics, and swimming behavior. The intergenerational effects may be due to direct contact of the F2 organism with contaminants at the stage of germinal cells, while transgenerational effects could reflect epigenetic modifications inherited from the F0 generation. Biochemical parameters appeared to be restored as the fish develop. Intergenerational exposure to pure glyphosate drastically reduced the ability of rainbow trout to face a viral infection, potentially due to the inability of fish to elicit an efficient antibody response. Our results demonstrated that glyphosate exposure induced both inter and transgenerational toxicity, sometimes with different effects depending on the physiologic functions considered. GBHs, particularly during early development, seemed to occasionally modulate the effects of the active substance. Re-exposure to glyphosate (i.e. multigenerational exposure) did not increase the toxicity compared to inter or transgenerational exposures. These results need to be strengthened by integrating more specific parameters allowing for an in-depth investigation of the mechanisms of glyphosate toxicity, the relationship between the active substance and the co-formulants, and also toxicity inheritance through generations, which will be helpful to adopt future regulations for the use of glyphosate.

Competing interests

The authors declare that they have no conflict of interest.

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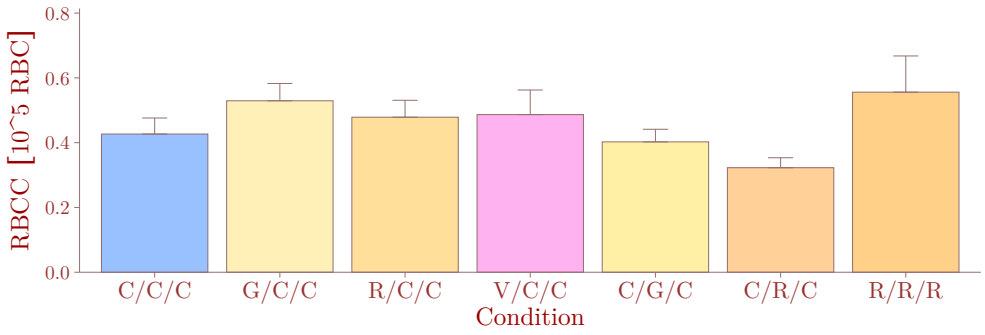
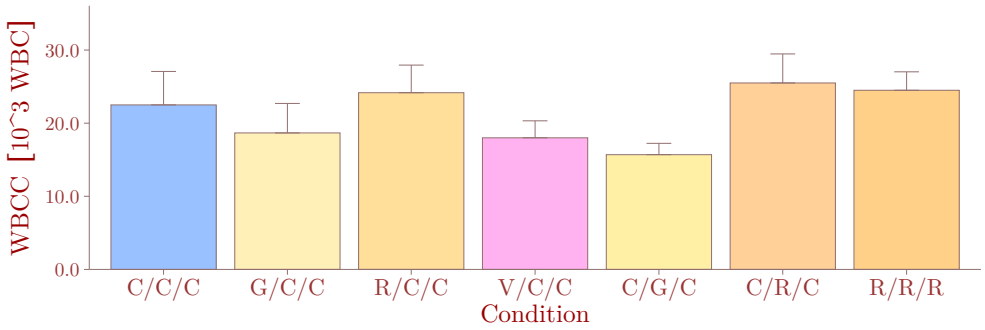
Appendix A. Blood cells counts in juvenile rainbow trout



(a) RBCC



(b) WBCC

Figure A.10: Barplot representing mean red blood cell count (RBCC) and mean white blood cell count (WBCC) as a function of exposure conditions (see Figure 2) 96 hours after the viral infection (S4). Standard errors are shown at the top of each bar ($12 \leq n \leq 13$).

Appendix B. Biochemical parameters in juvenile rainbow trout

Table B.6: Mean specific activities measured in gills of F2 juveniles (S4, see Figure 1 for graphical illustration) for the different chemical conditions (see Figure 2). Specific activities are expressed in IU mg^{-1} of protein. Standard errors are given in parentheses under each respective mean ($11 \leq n \leq 20$). The numbers in bold with an asterisk are significantly different ($p < 0.05$) from the values obtained for the control condition.

Parameter	**Mode of exposure**						
	Control	**Transgenerational**			**Intergenerational**		**Multigenerational**
	C/C/C	G/C/C	R/C/C	V/C/C	C/G/C	C/R/C	R/R/R
CCO	1.7 (0.085)	1.8 (0.096)	1.62 (0.083)	1.66 (0.077)	**2.11*** (0.14)	1.95 (0.062)	1.71 (0.067)
CS	0.42 (0.018)	0.43 (0.014)	0.42 (0.015)	0.43 (0.021)	**0.52*** (0.026)	0.46 (0.011)	0.44 (0.016)
CS:CCO	0.25 (0.0084)	0.24 (0.0073)	0.26 (0.0085)	0.26 (0.0108)	0.26 (0.0119)	0.24 (0.0073)	0.26 (0.0139)
GPx	0.04 (0.004)	0.046 (0.0029)	0.038 (0.004)	0.045 (0.0035)	0.052 (0.006)	0.044 (0.0044)	0.042 (0.0041)
LDH	27.94 (1.08)	27.49 (1.57)	26.05 (1.07)	27.58 (1.1)	31.21 (1.31)	30.79 (1.09)	27.93 (0.87)
LDH:CS	64.9 (2.65)	66.24 (3.05)	61.67 (2.42)	65.44 (2.61)	61.14 (2.11)	67.36 (2.35)	64 (3.22)

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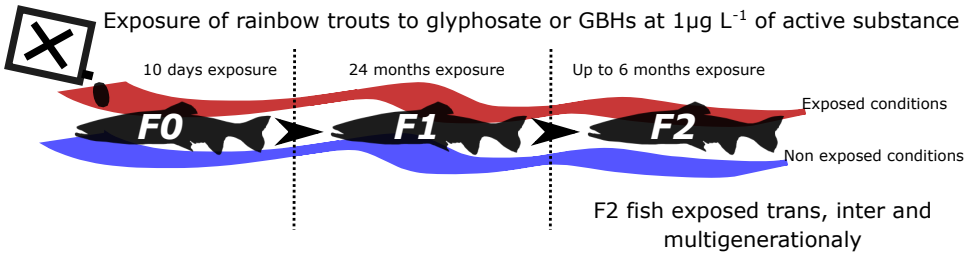
Graphical Abstract

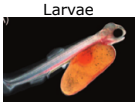
Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*

Jessy Le Du-Carrée, Rania Boukhari, Jérôme Cachot, Joëlle Cabon, Lénaïg Louboutin, Thierry Morin, Morgane Danion



F2 fish exposed trans, inter and multigenerationaly

Larvae



Biometrical defects
Metabolic modifications
Trans and integernerational behavior effects

Juveniles

Inter and multigenerational effects on viral susceptibility

Highlights

Generational effects of a chronic exposure to a low environmentally relevant concentration of glyphosate on rainbow trout, *Oncorhynchus mykiss*

Jessy Le Du-Carrée, Rania Boukhari, Jérôme Cachot, Joëlle Cabon, Lénaïg Louboutin, Thierry Morin, Morgane Danion

- Trans, inter and multigenerational toxicity of glyphosate was studied
- Pure and co-formulated glyphosate induced developmental toxicity on the F2 generation
- Inter but not transgenerational toxicity increased viral susceptibility of juveniles
- Both non-genetic inheritance and exposure at the germinal stage could be involved
- Re-exposure to parental contaminants did not seem to modulate toxicity