Multigenerational exposure to gamma radiation affects offspring differently over generations in Zebrafish

Noémie Guirandy, Beatrice Gagnaire, Virginie Camilleri, Isabelle Cavalie, Fabien Pierron, Patrice Gonzalez, Olivier Simon

Abstract

Mutigenerational studies are now of great interest in ecotoxicology and previous studies have shown the importance of conducting multigenerational studies when assessing radiation toxicity in fish. In our study, the first objective was to study the early life stages (embryo-larval stages) and critical functions such as reproduction (which are generally studied in the context of ecological risk assessment (ERA)), in order to assess its sensitivity. The second objective was to assess acquisition of phenotypic effects at some life stages over generations.

To our knowledge, this was the first time that irradiation of zebrafish (0.05 and 5 mGy.h-1) up to generation F2 was maintained with the following two exposure conditions: (1) recovery, only F0 genitors were irradiated and the progeny were placed in control condition, (2) irradiated condition, all generations were exposed. Multigenerational irradiation affected F1 parental reproductive capacity (reproductive success) mainly over the first reproductive cycle (104d) and larval survival rate. Unexpected yet significant effects on sex ratio were observed in F1 progeny after parental irradiation (mainly at 5 mGy.h-1).These effects were observed for both conditions -irradiated and recovery- suggesting transmitted effects from F0 genitor to offspring. All studied life stages were affected by ionizing radiation (IR), suggesting an alteration of vital physiological functions (reproduction and sexual determination). Such results highlight the hypothesis that IR affects population dynamics. In addition, the clear evidence of transmitted effects suggests worsening of effects at the population scale over generations. This approach is closer to environmental conditions to assess wild population fate, and thus highlights the importance of multigenerational studies in support ERA of ionizing radiation in fish.

Keywords: multigenerational, mortality, sex ratio, zebrafish, irradiation, transmitted effect

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1. Introduction

Exposure to ionizing radiation (IR) is reported to induce a variety of biological effects in fish (Anbumani and Mohankumar, 2012; Rhee et al., 2012). In most cases, the effects after gamma irradiation are assessed at specific sensitive stages, such as the embryo-larval stage, because they are considered to be most vulnerable to ionizing radiation (Gagnaire et al., 2015; Hu et al., 2016; Lerebours et al., 2020; Pereira et al., 2011; Praveen Kumar et al., 2017; Simon et al., 2011a). However, focusing on just one sensitive stage of life is not enough to obtain a comprehensive understanding of the effects ofirradiation in fish (Guirandy et al., 2019; Hurem et al., 2017a; Hurem et al., 2018). For example, at 50 to 53 mGy h^{-1}, F1 irradiated and non-irradiated

progeny from F0 irradiated zebrafish (*Danio rerio*) showed 100% mortality, providing an evidence of transmitted effects from parents to progeny and thus the importance of assessing multigeneration studies These dose rates are not environmentally relevant but contribute to the development of the dose-responses relationships necessary for ERA. Further studies at lower dose rates also need to be considered. At a lower dose rate (5 to 8.6 mGy h^{-1}), parental exposure led to significant effects at the molecular levels in progeny, via multiple processes, such as epigenetic mechanisms (Hurem et al., 2017b; Hurem et al., 2018; Kamstra et al., 2018). Moreover, multigenerational studies assessed over an entire life span should be done to represent at most the environmental conditions; indeed higher ecological radiosensitivity was observed in Chernobyl wildlife (Hazard dose rate affecting 50% of species at their 50% effect) compared to laboratory conditions.

The Chernobyl and Fukushima accidents released considerable amounts of radionuclides into the environment and have provided a strong impetus to better address the ecosystems affected by chronic exposure to gamma radiation (Bréchignac et al., 2016; Lerebours et al., 2016). However, traditional environmental risk assessment (ERA) approaches do not generally consider multigenerational exposure when predicting impacts on ecosystems. Therefore, classical ERA benchmarks might not be adequate for assessing irradiation effects in wild fish populations. Indeed, as for other pollutants, effects of IR are mainly assessed based on classical endpoints (reproductive success, fecundity, fertility, survival) measured at one stage of life, without considering the effects across multiple generations.The lack of data on toxicity after multigenerational exposure have encouraged the scientific community and policymakers to address laboratory multigenerational studies (EC-TG N°27, 2011), which has led to the development of the “Test No.443:

72 Extended One-Generation Reproductive Toxicity Study" (OECD, 2018) on fish. .
73 However, multigeneration studies on IR are scarce but they demonstrated that
74 multigenerational exposure can lead to harmful effects and highlights new toxic
75 mechanisms (see Guirandy et al. 2019). These studies suggested that IR can induce
76 (i) epigenetic alterations, such as DNA methylation located on gene promoters and
77 enhancers, which can be inherited by future generations and (ii) altered
78 transcriptomes.

79 Beyond studied parameters, selecting an appropriate exposure dose or dose rate
80 exposure is also very important to increase the relevance of ecotoxicity datasets.
81 Frequently, irradiation effects on fish focus exclusively on acute and short-term
82 exposures (Hu et al., 2016; Pereira et al., 2011; Tsyusko et al., 2011), which is not
83 environmentally realistic and not suitable for assessing chronic effects. Low dose
84 rates must also be studied to complete the dose/response relationship and more
85 precisely represent the environmental conditions. Ageneric screening value of 10
86 µGy h^{-1} has been defined as protected dose rate for terrestrial and aquatic
87 ecosystems (Garnier-Laplace et al., 2010). Environmental protection by International
88 Commission on Radiological Protection (ICPR) referred to the Derived Consideration
89 Reference Levels (DCRL), corresponding to a dose rate band where deleterious
90 effects can appear. A possible reduction in reproductive success for dose rates
91 between 40 and 4000 µGy h^{-1} was retained for freshwater fish (Reference Animals
92 and Plant: trout) (ICRP, 2012).

93 In this study, we investigated whether multigenerational gamma irradiation (^{137}Cs) of
94 zebrafish (*Danio rerio*) can affect the reproductive performances of animals and
95 induce effects on F1 and F2 progenies (Guirandy et al., 2019; Hurem et al., 2017a;
96 Hurem et al., 2018; Kamstra et al., 2018). We hypothesized that IR can influence

reproductive performance across generations. Therefore, a multigenerational experiment on fish covering three generations (F0 exposed at adult stage, F1 and F2) was performed at a low dose rate of 0.05 mGy h^{-1}, close tothe generic screening value (0.01 mGy h^{-1}, (Garnier-Laplace et al. 2006)) and lower DCRL range. A second dose rate of 5 mGy h^{-1} was studied to challenge the drastic effects on progeny previously determined in experiments focusing on high irradiated parental exposure (50 mGy h^{-1}) (Guirandy et al., 2019) and represents the upper dose rate of the DCRL. We used domesticated zebrafish (*Danio rerio* AB strain), which develop rapidly, have high fecundity, and are well described in the literature (Lawrence, 2007). *Danio rerio* is often used in ecotoxicology, for the assessment of stress effects in general and more recently for the characterization of multi-or transgenerational effects of stressors (Baker et al., 2014; Lin et al., 2020; Pierron et al., 2021; Siegenthaler et al., 2017; Simon et al., 2014). Moreover, zebrafish have been already used in studies assessing the effects of ionizing radiation (Epperly et al., 2012; Gagnaire et al., 2015; Houdigui et al., 2020; Hurem et al., 2017a; Hurem et al., 2018; Kamstra et al., 2018; Kong et al., 2016; Simon et al., 2011a). This study was designed to answer several questions: (i) How does gamma radiation impact different generations and how is this impact transmitted across generations? (ii) What are the sensitive endpoints to radiation at low doses or dose rate?

We reared F1 and F2 progenies from F0 adult irradiated for 30d. Two irradiation conditions were defined: (i) irradiated (I) where each generation was irradiated over time to observe any possible worsening of effects over the 2 generations and (ii) recovery (R) where only the F0 adult was exposed, progenies were in non-irradiated conditions to observe any possible parental transmission of effects. Adult reproductive performances, progeny survival, and progeny development were

evaluated and compared to previous experiments performed at a high dose rate (50 mGy h^{-1}) (Guirandy et al., 2019).

2. Materials and methods

2.1 Adult fish husbandry

Project #20995 was authorized by the Institut de Radioprotection et de Sûreté Nucléaire (IRSN) ethics committee no. 81 (EU 0520, C13-013-07) and complied with French regulations on performing experiments on animals in application of directive 2010/63//UE relating to animal protection. The study was conducted on wild-type zebrafish that were kept, reproduced, and irradiated in a zebrafish housing system (Zebtec Tecniplast Stand Alone) with recirculating oxygenated freshwater. Adult fish were acclimatized for 3 weeks to tap water + 20% demineralized water renewed daily (Aquadem; pH = 7.4 ± 0.4, conductivity = 398 ± 12 µS cm^{-1}, temperature = 28.4 ± 1.3 °C), with a 12:12-h light:dark cycle photoperiod. The fish were fed *ab libitum* three times a day with GEMMA Wean (Skretting®).

2.2 Fish rearing

The rearing method under controlled conditions was optimized based on different tests performed before the experimentation (choices of food, cleaning of devices, density of individuals, and water renewal for better survival), not presented here. From 0 hpf to 20 days post-fertilization (dpf), progeny were kept in crystallizing glass dishes (diameter 9 cm) in groups of 50 fish. The water used in crystallizing dishes was the same as that used for F0 adult fish. Crystallizing dishes were kept in an incubator (PANASONIC MIR-154), with nominal constant temperature of 28°C and a 12:12-h light:dark cycle photoperiod. From 15 dpf to 20 dpf, the water level in the crystallizing dishes was raised by 0.5 centimeter every day. Crystallizing dishes were cleaned daily. At 20 dpf, progeny were transferred to an aquarium (3.5L, Zebtec

Tecniplast Stand Alone) with a low water flow to avoid disrupting larvae locomotion. At adult age, water flow wasincreased to ensure appropriate water quality. From 7 dpf to 50 dpf, larvae were fed twice a day with 24h-old *Artemia salina* Nauplii and once a day with Gemma Micro ZF (Skretting®). Past this age, fish were fed *ad libitum* three times a day with GEMMA Wean (Skretting®) with a food dispenser. The same protocol was used for breeding the F2 generation from F1 adults.

2.3 Adult and embryo exposure

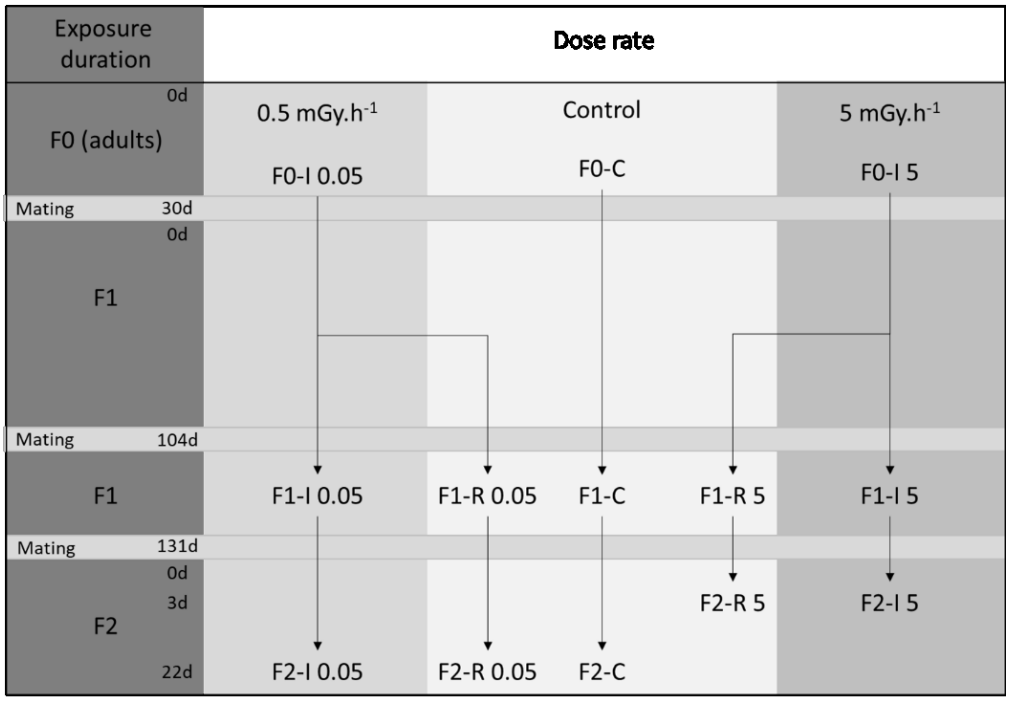

Figure 1: Experimental design and exposure conditions (duration (d) and dose rate (mGy h^{-1}) used for the multigenerational experiment. F0 adults were exposed over 30 days until reproduction. F1 progenies were then placed in irradiated (F1-I0.05, F1-I5) and non-irradiated (recovery, F1-R0.05, F1-R5) exposure conditions over 131 days. Mating was performed at 104 and 131d. F2 progenies (F2-I0.05, F2-I5) from F1 irradiated adults were irradiated over 22 days. F2 progenies (recovery, F2-R0.05,) from F1 recovery adult were placed in non-irradiated exposure over 22 days. F2 progenies (recovery, F2-R5) from F1 recovery adults were placed in non-irradiated exposure conditions over 72 hours. M: mating.

Nominal dose rates were 60–80 nGy.h^{-1} (control-C), 0.05 (I0.05) and 5 (I5) mGy h^{-1}. Gamma-rays were emitted from a ^{137}Cs source (444 GBq, 662 keV; IRSN, MICADO-

167 Lab platform). Dose rates were simulated using MCNP5 software and measured
168 using thermoluminescent dosimeters (Chiyoada Technologies), and the values
169 represented between 98% and 108 % of the nominal values. Control dishes were
170 kept in a separate room.

171 The population density of adult fish was 4 fish per liter. 30 couples of F0-adult fish per
172 condition (F0-C, F0-I0.05, F0-I5) were exposed over 30 days. Daily controls and
173 feeding were conducted as described in Guirandy et al. (2019). Adult mass (g, fresh
174 mass) was measured after dissecting the fish.

175 For F0 reproduction, F1 offspring were obtained from 15 spawning couples (30 fish, 1
176 female: 1 male) (i.e., replicates) per condition. Mating and viability were determined
177 as described in Guirandy et al. (2019). The embryos from 3 spawns per condition
178 were separated into 2 groups per spawn. The first one was kept in crystallizing
179 dishes with a density of 50 eggs per dish. The F1 embryos (F1-I0.05, F1-I5) from 3
180 spawns from across all 15 spawning couples were then positioned in an incubator
181 and irradiated at the same irradiation conditions as for F0. The second group (F1
182 recovery embryos (F1-R0.05; F1-R5)) was placed in non-irradiated conditions. F1
183 offspring were irradiated over 131 days. Several reproduction assays were initiated
184 between 104 and 131d to assess the reproductive capacity of all F1 fish. For the
185 reproductions performed at 104, 105, 111, 112d and 131d, the couples (1 female: 1
186 male) were formed from the observation of secondary sexual characteristics. During
187 the last reproductive cycle carried out at 131d, the sexes were checked during the
188 dissection of the fish.

189 For F1 reproduction, there were at least 8 spawning couples (1 female: 1 male) per
190 spawn and condition, except for F1-I5 (n=5). F2 offspring was then kept in the same
191 conditions as F1 embryos until 22 days (F2-I0.05 F2-R0.05) of exposure and until 72

hours (F1-I5 and F2-R5) of exposure. F2-I0.05 and F2-I5 came from the irradiated F1 generation. F2-R0.05 and F2-R5 came from the F1 recovery condition, where only the F0 adults were irradiated. All fish from irradiated conditions were reproduced under irradiation.

2.4 Ecologically representative endpoints for adults and for progeny

Reproductive success (number of couples that spawned), the fecundity (number of eggs per female) of adults (F0 and F1) and the quality of 4 hpf-eggs were assessed.

For each generation (F1 and F2), the progeny survival rate (%) was assessed daily until the stage with no more death (22 dpf) for all conditions. Three technical replicates of 50 eggs originating from 3 different spawns from across all 15 spawning couples per condition were tested. Survival rate was presented for 3 stages: 4 dpf, 8 dpf and 22 dpf. They were chosen because 4 dpf is a commonly studied stage for ecotoxicity bioassays; 8 dpf is a critical stage that corresponds to the beginning of the self-feeding period without a yolk sac, and 22 dpf is the stage at which spontaneous embryo mortalities seizes.

For the F1 generation, the male-female distribution was assessed based on observable sexual characteristics for the remaining individuals (n: F1-C = 194; F1-R0.05 = 124; F1-R5 = 136; F1-I0.05 = 127; F1-I5 = 119).

2.5 Theoretical population size

Theoretical fish production was estimated for the F1 and F2 generations and for each condition. At the beginning, there were 60 individuals per condition for the F0 generation. The total effective population was calculated with the product of number of females, reproductive success rate, mean number of viable eggs and survival rate of progeny (at 22 dpf or 72 hpf for F2) (Simon et al., 2011b). Error bars correspond to

incertitude from mean number of viable eggs number and survival rate: $\Delta TP = TP\left(\frac{\Delta \text{Mean number of eggs}}{\text{Mean number of eggs}} + \frac{\Delta \text{ survival rate}}{\text{survival rate}}\right)$.

2.6 Statistical analysis

All data are presented as mean values ± SD, with significance taken as $p < 0.05$. For both F0 and F1 masses, conditions were compared using Anova with BoxCox transformation when normality and homogeneity were not verified. Concerning data relating to the F0 generation, conditions were compared using a GLM (Generalized Linear Model, glm function in R). Poisson and binomial distributions were selected for fecundity and reproductive success parameters respectively. Concerning data relating to the F1 and F2 generations, conditions were compared using a GLMM (Generalized Linear Mixed Model, glmm function in R). This model integrates the non-independence of data. Our data are linked by spawn, corresponding to the three spawns chosen for breeding embryos. The spawn was chosen as a “random” effect. The binomial distribution was used to analyze all the parameters (reproductive success, sex ratio, survival rate) expect for the fecundity parameter for which a Poisson distribution was preferred. For reproductive success of F1 131 dpf, as the dataset represented can be defined as quasi-complete separation, because of zero values, the invariant Jeffreys prior method was used with the brglm package. The analyses were performed using R software (R Core Team, 2013) with the following packages : “tidyverse”, “here”, “knitr”, “lme4”, “MASS”,”car”.

3. Results

Regardless of treatment groups, no adult fish mortality was observed during the experimental period.

3.1 Cumulative doses after exposure conditions

Measured dose rates (0.051 ± 0.002 and 5.15 ± 0.3 mGy h^{-1}, n=10) were close to nominal dose rates. For the F0 generation, cumulative doses, based on nominal dose rate, in adults ranged between 0.036 and 3.6 Gy (Table 1). Higher cumulative doses (0.16 and 15.7 Gy) were measured after 131d of F1 exposure.

Table 1: Cumulative doses (Gy) calculated from the nominal dose rate for each condition (control, dose rate of 0.05 and 5 mGy h^{-1} (I) and Recovery (R) and each generation (F0, F1, F2).

	Cumulative dose (Gy)			
Dose rate (mGy h^{-1})	**F0**	**F1**	**F2**	
	30d	**131d**	**72h**	**22d**
Control	5.8E-05	2.5E-04	5.8E-06	4.2E-05
I 0.05	3.6E-02	1.6E-01	3.6E-03	2.6E-02
I 5	3.6E+00	1.6E+01	3.6E-01	-
R 0.05	-	2.5E-04	4.2E-05	4.2E-02
R 5	-	2.5E-04	5.8E-03	-

3.2 Adult reproductive performances

*Table 2: Mass (g, fresh weight), reproductive success (%) and fecundity of F0 and F1 adults after exposure to control (C), 0.05 and 5 mGy h^{-1} (I) and Recovery (R) conditions * (p<0.05), ** (p<0.01), *** (p<0.001).*

Condition	Age at reproduction (days)	Mass (fw, g)		n	Number of couples	Reproductive success (%)	Fecundity	Survival at 4 hpf (%)
		Male	Female					
F0 – C		0.59 ± 0.10	0.76 ± 0.21	28	30	57	362 ± 175	90.67 ± 12.48
F0 – I 0.05	**270**	0.53 ± 0.09	0.76 ± 0.21	27	30	47	247 ± 127*	99.90 ± 0.27
F0 – I 5		0.53 ± 0.06	0.64 ± 0.17	29	30	43	206 ± 128**	95.90 ± 15.40
F1 – C		-	-		20	75	178 ± 85	97.56 ± 5.72
F1 – I 0.05		-	-		32	53**	271 ± 152	97.30 ± 4.13
F1 – I 5	**104**	-	-		20	15***	243 ± 46	63.60 ± 13.28
F1 – R 0.05		-	-		20	35***	80 ± 95	81.63 ± 21.16
F1 – R 5		-	-		29	10***	179 ± 192	97.50 ± 2.20
F1 – C		0.43 ± 0.06	0.62 ± 0.17	8	15	80	399 ± 223	92.42 ± 21.62
F1 – I 0.05		0.40 ± 0.09	0.49 ± 0.12	8	15	67	298 ± 162	99.56 ± 0.53
F1 – I 5	**131**	0.52 ± 0.05	1.02 ± 0.41	5	5	60	245 ± 233	99.22 ± 0.77
F1 – R 0.05		0.47 ± 0.06	0.61 ± 0.14	8	15	100	283 ± 153	92.49 ± 24.68
F1 – R 5		0.55 ± 0.14	0.85 ± 0.17	8	12	33*	291 ± 204	81.37 ± 26.82

Adult genitors showed relatively homogeneous masses for both males and females for generations F0 and F1 (Table 2). Concerning F0 reproduction, the reproductive success (RS) of F0 - I5 adults was lower (43%), but not significantly different compared to the control (57%). The egg quality expressed by the survival rate at 4 hpf was not impacted by gamma irradiation (p.val>0.05), however, fecundity was impacted (p.val<0.05). Concerning F1 reproduction, differences were observed between the two reproductive tests. For the first reproduction test (104d), the RS for all irradiated conditions were lower compared to control conditions, in particular for F1-I5 (15%) and F1-R5 (10%), which were significantly different from the control values. For the second reproduction test (131d), F1 - I0.05 and F1 - R0.05 showed a RS similar to F1-C, but with a high reproduction for F1-R0.05 (100%). RS in the F1-R5 group (33%, n=12) was significantly different from control values (80%, n=15), and there was a decreasing trend compared to the F1-I5 (60%, n = 5) group, although this was not statistically significant. Also, for this generation, no significant difference was observed regarding the fecundity among treatment groups.

Egg quality (survival at 4 hpf) was greater than 90% and was not impacted by exposure to IR; however a decreasing trend was observed for the first reproductive cycle of F1-I5 (63.6%), F1-R0.05 (81.6%) and for the second reproductive cycle of F1-R5 (81.4%). High variability among couples was observed, which reduced statistical power and is likely the reason for the lack of significant effects. Gamma irradiation could lead to a decrease in RS (from -10% to -60%). Note that for the 2nd reproductive cycle, females from F1-I5 were as few as 5 because only 5 females were able to reproduce from the tested adult fish.

No relationship was observed between egg quality, fecundity and RS.

3.3 Larval stage

3.3.1 For F1

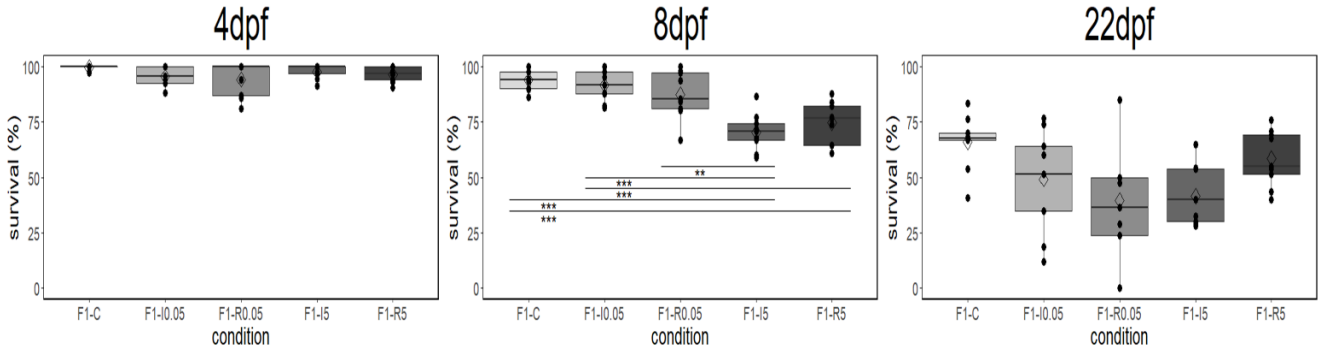

*Figure 2: Box-plot of survival rate of progeny (%) over time tor the 1st generation (F1) at 4, 8 and 22 dpf after control (C), dose rate exposures to 0.05 and 5 mGy h^{-1}(I) and recovery (R) conditions. The boxplot represent the 25^{th} and the 75^{th} percentile with the median indicated by blackline. Dots represent individual data. Means are indicated as empty lozenge. For each condition, n = 9, * (p<0.05), ** (p<0.01), *** (p<0.001).*

At 4 dpf, the average percentage of survival observed was high and between 94.2 and 99.7% for all exposure conditions (Figure 2). No significant difference was observed among treatment groups when comparing more than 2 conditions. However, a high individual variability was observed for F1-R0.05 group (SD = 7.66). At 8 dpf, the average percentage of survival in F1-I5 (70.4%) and F1-R5 (74.6%) conditions was significantly lower than the controls. A trend towards increased survival rate was observed for F1-R5 vs F1-I5. At 22 dpf, the average percentage of survival observed was 66.0, 49.2, 39.6, 41.5 and 58.5% for F1-C, F1-I0.05, F1-R0.05, F1-I5 and F1-R5, respectively and showed high individual variability (SD between 12.4 and 31.2), which may explain the lack of statistical differences among conditions. The highest variability for F1-R0.05 occurred in two replicates. Finally, the

decrease in survival rate over time was more significant for irradiated conditions than control conditions. No further mortalities were observed after 22dpf until 131dpf.

3.3.2 For F2

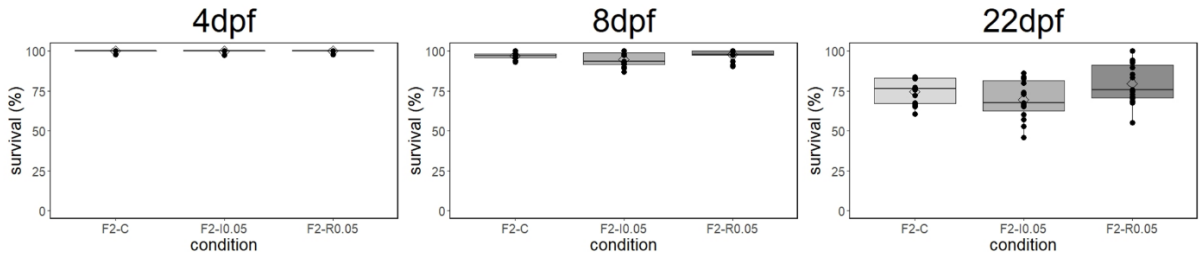

*Figure 3: Box-plot of survival rate of progeny (%) over time tor the 2nd generation (F2) at 4, 8 and 22 dpf after control (C), dose rate exposures to 0.05 and 5 mGy h^{-1}(I) and recovery (R) conditions. The boxplot represent the 25th and the 75th percentile with the median indicated by blackline. Dots represent individual data. Means are indicated as empty lozenge. For each condition, n = 15, * (p<0.05), ** (p<0.01), *** (p<0.001).*

Concerning F2 generation survival (reproduction at 131d), the individual range of variability was low for the first stages (Figure 3) but increased at 22 dpf; however variability was clearly lower than for the F1 generation. No significant difference of survival rate was observed among F1-I0.05, F1-R0.05 and control values. The effect of irradiation in the F2 generation appeared less noticeable than that measured in the F1 generation. The survival rate (%) was only assessed at 3 dpf for F2-I5 (53.3%, n=3 breeding pairs) and was lower than that measured for the first reproductive cycle (data not shown).

3.4 Adult stage of F1 generation

3.4.1 Sex ratio

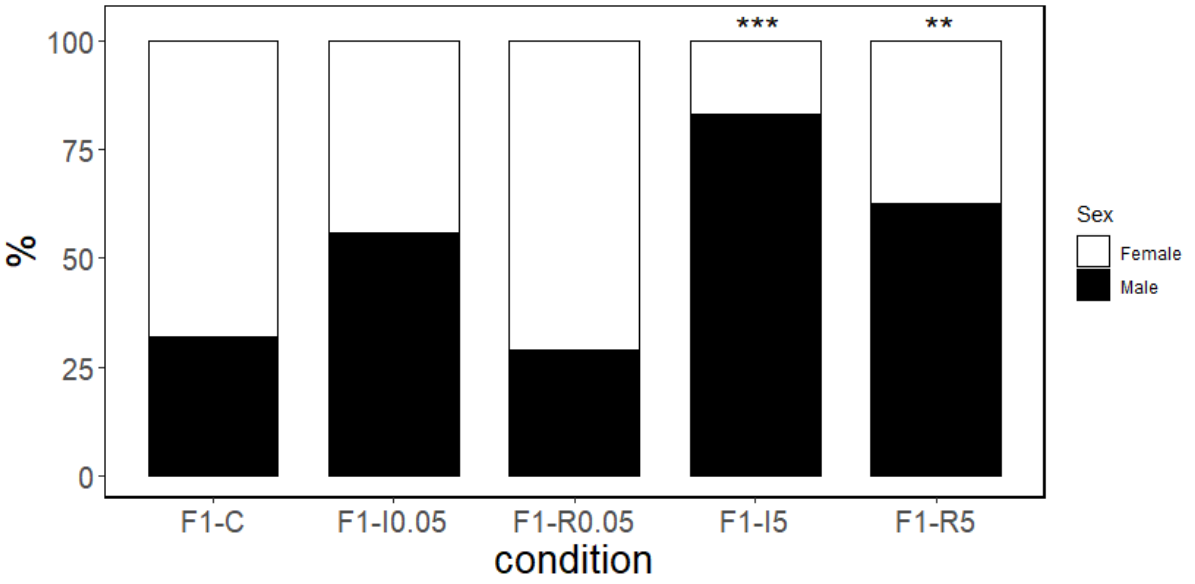

*Figure 4: Female and male F1 adult distribution (%) a in control (C), 0.05 and 5 mGyh^{-1} irradiated (I) and in recovery (R) conditions. at 131 days. Total number of adult fish; F1-C = 194; F1-R0.05 = 124; F1-R5 = 136; F1-I0.05 = 127; F1-I5 = 119. * (p<0.05), ** (p<0.01), *** (p<0.001).*

Significant differences were observed concerning the male and female distribution for the F1 adults (Figure 4). Greatest female (%) disruption was for F1-I5 (17%, 20 females) and F1-R5 (37%) compared to F1-C (68%). No difference was observed between F1-I5 and F1-R5, whereas a significant difference was seen between F1-I0.05 and F1-R0.05. The percentage of females was higher for F1-R0.05 than for F1-I0.05 and showed a comparable pattern to F1-C (68%).

3.5 Population size

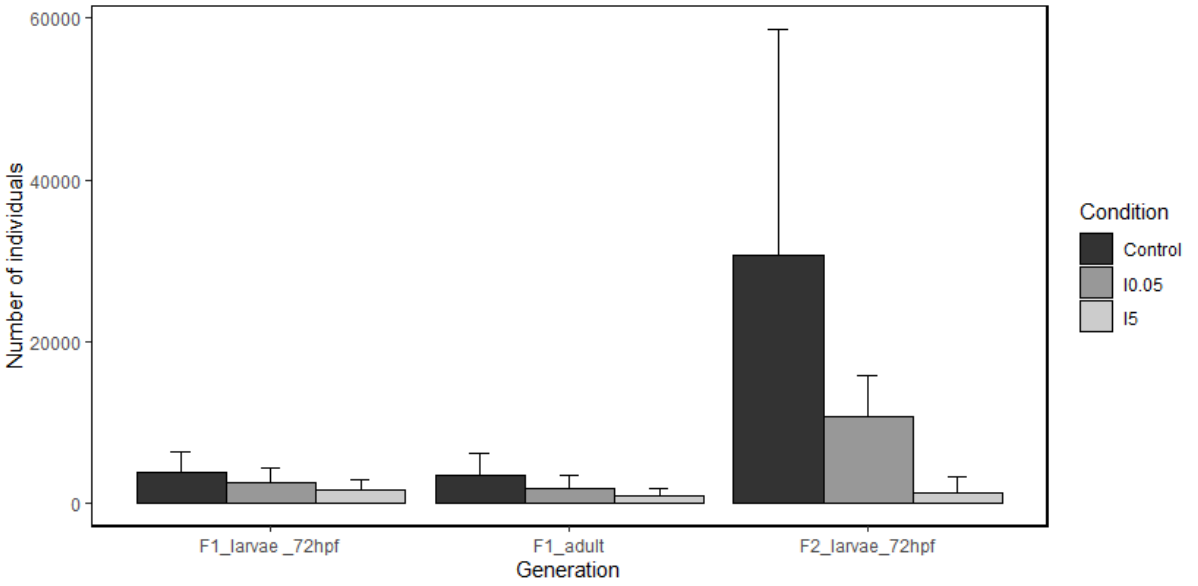

Figure 5: Estimation of population of F1 and F2 generations after irradiation exposures (Control conditions (C), to 0.05 and 5 mGy h^{-1} (I). At the beginning, 60 fish were present (sex ratio = 1 male: 1 female) per condition for the F0 generation. The total effective population was calculated with the product of number of females, reproductive success rate, fecundity (mean number of viable eggs) and the survival rate of progeny (at 3 and 22 dpf for F1 and 3 dpf for F2).

For the F1 generation, the average population of F1-C (3493 individuals) was higher than F1-I0.05 (x2), and F1-I5 (x3.8) (Figure 5). At 72 hpf, data were identical to 22 dpf for F1 – C (22 dpf: 3493; 72 hpf: 3715). Thus, it was decided to use survival at 72 hpf to include a condition with 5 mGy h^{-1} irradiation in the description of variation in population. For the F2 generation, the theoretical population of F2-C (30701 individuals) was much higher than that of F2-I0.05 (x2.9) and F2-I5 (x26.3). Note that for F2-I5, RS was done only for females previously able to reproduce. The most severe effects were observed for condition I5 while a slight improvement was observed for condition I0.05 between the two generations. By projecting the values of endpoints measured in the second generation, up to the third generation, the number

348 of adult fish is 12 times lower for condition I0.05 and more than 600 times lower for
349 condition I5 compared to control conditions.

350 4. Discussion

351 After gamma irradiation exposure to 0.05 and 5 mGy h-1 over two generations,
352 adverse effects were shown in F1 generation. All life stages were affected with early
353 mortality, poor RS in first reproduction and altered male biased sex ratio.

354 *4.1 Multigenerational effects*

355 Significant decreases in reproductive success (RS) (30% for F1-I0.05, 80% for F1-I5
356 compared to F1-control conditions) were observed for the first F1 reproductive cycle
357 (104d) compared to the F0 reproductive cycle (18% for F0-I0.05, 25% for F0-I5,
358 compared to F0-control). Only 15% of all F1-I5 couples was able to reproduce with
359 low egg quality (64±13%).

360 Reproduction was tested at 104d, an age greater than the age of sexual maturity
361 (90d) in the Danio ((Lawrence, 2007). Since all fish were under the same
362 reproductive conditions, the hypothesis was that irradiation during the entire oocytes
363 development cycle of F1 adults would impair reproduction. This is also justified by the
364 fact that between the first reproduction (104 days), for which all mature oocytes have
365 been expelled, and the second reproduction (131 days), for which the mature II
366 oocytes were irradiated only for 27 days, an improvement of RS was observed;
367 however, RS of irradiated F1 adults was still lower than the control values (Table
368 2)..Effects on reproductive capacities have already been observed for other
369 contaminants such as endocrine pollutants (Li et al., 2019), which are widely studied,
370 when compared to IR. Since nuclear power plants are widely used for energy
371 production, it appears necessary to study the impacts of radionuclide releases more
372 extensively. As effects are observed even from low doses, with a decrease in

reproductive success, the question of the consequences on population dynamics may arise. For zebrafish, the irradiation time for developing mature oocytes is short (27 days) and this results in an insignificant decrease in RS. In contrast, after 104 days of irradiation of mature oocytes, a significant decrease in RS was observed. For wild fish species, which often have a long maturation time with just one reproduction per year, a decrease in reproductive success could have severe consequences.

A significant effect on F1-I5 (70%) and F1-R5 (75%) survival rates was observed at 8 dpf compared to control conditions (90%), a key stage of development (Geffroy and Simon, 2013; Lawrence, 2007) whereas no effect was observed when embryos from unexposed parents were chronically irradiated at this dose rate (Gagnaire et al., 2015; Houdigui et al., 2020; Hurem et al., 2017b; Simon et al., 2011a). The same was found for other species of fish (Guppy embryos (up to 8.4 Gy) or mosquito fish (12-50 Gy)) (ICPR, 2008). Mortality for *D. rerio* larvae was only observed after acute and short-term irradiation (10 Gy, 1.16 Gy/min) (Pereira et al., 2011; Praveen Kumar et al., 2017). At 8 dpf, the survival rate at the lower dose rate was identical to the control group, suggesting a tolerance to lower dose rates. We hypothesize that no significant effect was observed at 5 mGy h^{-1} and 22 dpf because of high individual variability among F1 individual eggs. Number of individuals and technical replicates were sufficient but eggs originated from only 3 genitors, and some genitors produced eggs of poorquality. Moderate effects were still observed even when progeny was not irradiated. However, these results were different to those observed previously, although a comparable trend with a decrease of survival rate had been observed at 22d (5 mGy h^{-1}, (Guirandy et al., 2019)). We hypothesized that this difference may be a function of differences in sensitivity of the batches of fish (backgroung of different genitors, age, strains) used for the different experiments. For the second generation,

F2-I5 showed a low survival rate (53%; 3dpf) obtained only from 3 spawns.Additional investigations will be necessary to confirm the trend in survival rate observed for F1-I0.05 and F1-R0.05.

Results indicated that parental exposure had consequences on the survival rate of progeny, as also observed for other biological models (Buisset-Goussen et al., 2014; Gilbin et al., 2008; Parisot et al., 2015). Parental exposure could be considered as a critical window of sensitivity in F1 development (Ivy et al., 2017) since effects were observed at the phenotypic scale but also at the molecular scale after genitor irradiation (Hurem et al., 2017a).

For ERA, life stage specific sensitivity must be considered alongside exposure conditions to define threshold values. Here, effects at 5 mGy.h-1 were significant but much less than at 50 $mGy.h^{-1}$ (Guirandy et al., 2019). So, it would be worthwhile to conduct experiments with dose rates between 5 and 50 $mGy\ h^{-1}$ to confirm this gap in survival rate after parental irradiation.

No mortality was observed from 22 to 104/131 dpf, suggesting that direct or indirect (transgenerational) effects of IR only affected early development stages. The results confirm the sensitivity of early embro-larval stage of *D. rerio* after parental exposure.

Unexpected effects were observed at the highest dose rate (5 $mGy\ h^{-1}$). Selection of couples based on secondary sexual characteristics had been proven to be difficult, and when sex was determined after dissection of adults, the sex ratio was significantly biased in favor of males, with 4 and 1.8 times lower numbers of females for F1-I5 and for F1-R5, respectively. Irradiation could affect sex differentiation as previously observed after exposure to hypoxia, high temperature and pollutants in zebrafish (Brion et al., 2004; Pierron et al., 2021; Valdivieso et al., 2020; Wang et al., 2011). Exposure to heat in fish is usually known to increase the number of males.

The zebrafish housing system (Zebtec Tecniplast Stand Alone) used recirculating oxygenated freshwater that prevents hypoxia and temperature variation. Responses to environmental changes can be mediated by epigenetic mechanisms as discussed by Pierron et al. (2021) and Valdivieso et al. (2020). Significant effects were obtained without progeny irradiation (F1-R5) but were higher when the progeny were also irradiated (F1-l5). F0 adult irradiation affected the non-exposed F1 generation. The sex ratio expected for zebrafish reared under control laboratory conditions is theoretically 50:50 (female to male) or with a small predominance of males (40:60) (Santos et al., 2017; Simon et al., 2014). This was not the case in our study, where females were predominant (68%). Previous studies have shown that sex ratio was linked with maintenance conditions, such as temperature, density and nutrition (Pierron et al., 2021). In our case, control and recovery group were maintained under the same conditions. Therefore, the significant difference in the sex ratio between these two conditions can be attributed to the irradiation and not to the bias of the sex ratio of our population.

In gonochoric species such as zebrafish, the gonads are "ovary-like" before genetic determination and then differentiation into male or female gonads. Since zebrafish sex is determined by genetic factors (genetic sex-determination (GSD)) and irradiation targets the genome, larval mortality and disruption of sex ratio effects at 5 mGy h-1 could be induced by genetic mechanisms.

Moreover, zebrafish sex determination could be influenced by environmental factors (environmental sex-determination (ESD)) such as hypoxia, temperature, EDCs, population density, and food (Santos et al., 2017; Valdivieso et al., 2020). The precise mechanisms of these environmental factors are not understood, but studies suggest that the endocrine stress-axis could play a critical role. In medaka, a GSD

448 species, temperature induces masculinization through an increase in cortisol
449 (Fernandino et al., 2012; Hayashi et al., 2010). It has also been shown that cortisol
450 was able to induce the masculinization of both behavioral and morphological traits of
451 female *Gambusia affinis* (Geffroy and Bardonnet, 2016).Moreover, many studies
452 have pointed out that cortisol is able to alter the production of gonadal steroids,
453 because the enzymes involved in their synthesis (11-βHSD) are also involved in
454 producing/inactivating glucocorticoid. Cortisol suppresses the brain-pituitary-gonadal
455 (BPG) axis in females, leading to lower pituitary gonadotropin content, reduced
456 plasma sex steroid levels, and decreased gonadal weight (Tovo-Neto et al., 2020). In
457 trout (*Salmo gairdneri*), adding cortisol to water during sexual differentiation triggered
458 testis differentiation and leads to a male-biased population. Moreover, cortisol
459 inhibited aromatase production, which in turn resulted in male-biased offspring.
460 Aromatase expression and/or activities in zebrafish have been shown to be disrupted
461 by environmental pollutants (Hinfray et al., 2018). Since RI at high dose rates
462 produce a stressful environment, we hypothesized that RI can stimulate cortisol
463 production, which is the stress hormone produced under stressed environment.
464 These observations strongly encourage us to measure the level of cortisol.

465 Cumulative effects on RS, sex ratio and survival rate observed at 5 mGy h^{-1} led to a
466 significant decrease in effective population (x26.3). Note that the impact on the
467 population was calculated from F1 effects observed during the second reproductive
468 cycle. Greater effects on the F1 generation observed during the first reproductive
469 cycle led to a more significant decrease in effective population. Effects on the F2
470 population only affected the survival rate (72hpf), which decreased compared to F1
471 and should be confirmed with more replicates and life stages since we stopped the
472 experiment at 72hpf. Beyond the decrease in effective population, disturbing the sex

473 ratio can have major consequences for mating competition and success, and on the
474 behavior of territorial males and female aggressiveness. The sex ratio for the R5
475 condition was also significantly biased, leading to an imbalance in the population. At
476 0.05 mGy h^{-1}, an insignificant decreasing trend in the population size was observed.

477 Multigenerational exposure was also used to assess the worsening of effects over
478 generations. The decrease in RS compared to control values was observed over the
479 two generations in comparable proportions (I0.05: 0.82; I5: 0.75, table 2) between F0
480 and the second F1 reproduction. The worsening of the effects of irradiation on this
481 parameter for both dose rates was observed when F1 reproduced for the first time.
482 Concerning the fitness of the larval stage, the F2-I5 survival rate was low and only
483 evaluated at 3 dpf. Effects appears earlier during development for F2 generation
484 compared to F1 generation, highlighting a worsening effect. Since these results were
485 preliminary, effects on F2 survival should be confirmed . For the lower dose rate, no
486 effect on F2-I0.05 survival rate was observed, rather indicating an improvement
487 despite irradiation. Finally, the disruption of the sex ratio confirms the worsening of
488 the effects between the 2 generations. Future studies should determine sex in the F2
489 generation to confirm this effect.

490 The survival rate showed slight improvement under the recovery conditions. Note that
491 recovery fish were only exposed during the F0 generation. Although no significant
492 difference was observed, the survival rate was slightly better for F1-R5 than for F1-I5.
493 This observation could not be confirmed for 0.05 mGy h^{-1} because of the high levels
494 of variability for the F1-R0.05 condition. It would be worth assessing potential repair
495 mechanisms at the molecular scale between these two types of exposure scenarios.
496 Reversible effects could also be explained by epigenetic mechanisms. However,
497 some epigenetic marks appeared to persist over multiple generations at 8.7 mGy h^{-1}

498 (Kamstra et al., 2018). Molecular effects, due to their high sensitivity, could persist
499 longer while phenotypic effects are more prone to recover over time.

500 ***4.2 Irradiation-impacted stages of life or physiological functions***

501 The recovery condition was studied to show differences between irradiated and non-
502 irradiated progeny born from irradiated couples. It should be kept in mind that the
503 irradiation of early stages from parents not exposed to these dose rates does not
504 affect the survival of the embryos (Gagnaire et al., 2015; Guirandy et al., 2019;
505 Houdigui et al., 2020; Hurem et al., 2017a; Simon et al., 2011a). Parental exposure
506 led to great or moderate effects on survival rate at 50 (Guirandy et al., 2019) and 5
507 mGy h^{-1} (this study) of F1 progeny, respectively. This confirms the sensitivity of this
508 biological stage. However, as survival rate and sex ratio were also affected in the
509 recovery condition, we can hypothesize that the irradiation mainly affects late
510 gametogenesis in adults, where it leads to effects on progeny after exposure to 5 and
511 50 mGy h^{-1}. Stage III of oogenesis is the process of vitellogenesis, in which the
512 oocyte begins to incorporate Vtg and several maternally-transferable compounds
513 (Faught and Vijayan, 2018). The latter are involved in various key processes such as
514 cortisol and thyroid hormone regulation, immunological responses, endocrine stress
515 axis development, epigenetic (*de novo* DNA methyltransferases) and post-
516 transcriptional (miRNA pathway components and specific miRNAs) regulation of
517 gene expression (Vera-Chang et al., 2019). The alteration of these maternally-
518 transferable compounds, as observed for different molecular markers (Guirandy et
519 al., 2019; Hu et al., 2016; Hurem et al., 2017a; Kamstra et al., 2018) after parental
520 irradiation can have late repercussions on zebrafish development.

5. Conclusion

This paper investigated the effects of gamma irradiation on *Danio rerio* after multigenerational exposure to two dose rates (0.05 and 5 mGy h^{-1}). It needs to be acknowledged that the Danio model cannot represent all fish species, especially with regard to effects on reproductive processes. This study completed a previous study that focused on the effects of a high dose rate (50 mGy h-1) on the same biological model. The results obtained provide comprehensive insights into the diversity of the responses to gamma irradiation dose rates. Moreover, this study answered many questions concerning irradiation methods that should be taken into account in future studies: (i) Multigeneration exposure shows The survival rate showed slight improvement under the recovery conditions that each generation was impacted differently or was not impacted at the phenotypic scale. Irradiation (0.05 and 5 mGy h^{-1}) may affect different life stages (adult: reproductive success, sex ratio and larval mortality). These findings emphasize an impact on some physiological functions (gametogenesis, sexual determination). Such effects can also affect population dynamics. Further experimentation is required to confirm these results. Moreover, due to the diversity of responses from one dose to another, it is necessary to study a wide panel of doses.

(ii) Multigenerational exposure makes it possible to acquire data on the reproductive capacities of adults exposed throughout their lifespan and on the fitness of the embryo-larval stages. These data, with high ecological value, can be used to roughly assess population dynamics and the worsening (or not) of the effects. Performing assays that assess effects of IR at different biological stages of *Danio rerio* separately could provide less realistic information than a single multigenerational assay.

Since wild populations are suspected to be more sensitive to radiation than laboratory populations, this could partly be explained by worsening effects after exposure over generations although model species have a lower polymorphism than wild species.

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