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Reproductive Capacity, but not Food Consumption, is Reduced by Continuous Exposure to Typical Genotoxic Stressor γ -Rays in the sentinel species *Gammarus fossarum*

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1 Reproductive capacity but not food consumption is reduced by continuous
2 exposure to the typical genotoxic stressor γ -rays, in the sentinel species
3 *Gammarus fossarum*.

4

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11

12 HIGHLIGHTS

- 13 • The impact of chronic exposure to ionizing radiation was assessed in male and female gammarids.
- 14 • Ionizing radiation affects the reproduction of *Gammarus fossarum*, that is critical for population
15 dynamics.
- 16 • Food consumption, which is linked to the general health status of gammarids, is not reduced by
17 ionizing radiation.
- 18 • Radio-induced reprotoxicity is more severe in male than female gammarids after chronic exposure.

19

20 ABSTRACT

21 The long-term impact of radiocontaminants (and the associated risks) for ecosystems are still subject
22 to vast societal and scientific debate while wildlife is chronically exposed to various sources and
23 levels of either environmental or anthropogenic ionizing radiation due to the use of nuclear energy.
24 This study aims to assess induced phenotypical responses in both male and female gammarids after
25 short-term continuous γ -irradiation, acting as a typical well characterized genotoxic stressor that can
26 interact directly with living matter. In particular, we started characterizing the effects using
27 standardized measurements for biological effects on few biological functions for this species,
28 especially feeding inhibition tests, molting and reproductive ability, which have already been proven
29 for chemical substances and are likely to be disturbed by ionizing radiation. The results show no
30 significant differences in terms of the survival of organisms (males and females), of their short-term
31 food consumption which is linked to the general health status (males and females) and of the molting
32 cycle (females). In contrast, exposure significantly affected fecundity (number of embryos produced)
33 at the highest dose rates for irradiated females (51 mGyh⁻¹) and males (5 and 51 mGyh⁻¹). These

34 results showed that, in gammarids, reproduction, that is a critical endpoint for population dynamics,
35 is the most radiosensitive phenotypic endpoint, with significant effects recorded on male
36 reproductive capacity, which is more sensitive than in females.

37

38 INTRODUCTION

39 In the environment, wildlife is chronically exposed to various sources and levels of ionizing radiation,
40 natural or anthropogenic due to the use of nuclear energy (civilian or military use, accidental release,
41 and side activities) (UNSCEAR 2000). Natural ionizing radiation includes cosmic rays and terrestrial
42 radiations coming from natural elements present in environments, such as uranium, thorium series
43 and potassium (^{40}K). After accidental release (e.g. Chernobyl, Fukushima), the levels of ionizing
44 radiation are mainly due to persistent radioisotopes such as ^{137}Cs , ^{90}Sr and plutonium isotopes, in
45 terrestrial and aquatic habitats. Whatever their decay process, radiocontaminants are harmful to
46 organisms in the environment due to their radioactivity, referred to herein as ionizing radiation (IR).
47 Indeed, IR can interact directly (by the ionization of biomolecules) or indirectly (by water radiolysis
48 and the production of reactive oxygen species) with DNA and other biomolecules, and cause damage
49 at different biological levels (UNSCEAR 2008). Characterization of such chronically induced biological
50 effects in different ecosystems and their representative non-human taxa, is crucial to define relevant
51 levels of radiological protection, to assess and predict ecological risk and then minimize impacts.

52 Since the Rio conference in 1992 and the publication of the Convention on biological diversity, the
53 International Atomic Energy Agency (IAEA) and the International Commission on Radiological
54 Protection (ICRP) have started to propose methodologies and reference values for radiological
55 protection of non-human species. Some reference levels have been proposed for different
56 ecosystems, i.e. generic-, terrestrial-, aquatic- one, ranging from $10 \mu\text{Gy}\cdot\text{h}^{-1}$ (Garnier-Laplace et al.,
57 2010) to $400 \mu\text{Gy}\cdot\text{h}^{-1}$ (UNSCEAR 2008; IAEA 1992) to assist in assessing the risk. In addition, 'Derived
58 Consideration Reference Levels' (DCRLs) have been defined by International Commission of
59 Radiological Protection (ICRP) for 'reference animals and plants' (RAPs) (ICRP 2008). However, the
60 available RAPs do not consistently represent the diversity of the species in the ecosystems
61 (Spurgeon, Lahive et al. 2020). In particular, crustaceans and their radio-sensitivity are currently
62 considered as insufficiently described and characterized. For instance, while the radio-sensitivity of
63 the parthenogenetic *Daphnia magna* has been quite well described, this is not true for crustaceans
64 with a sexual reproductive strategy (Dallas, Keith-Roach et al. 2012; Fuller, Lerebours et al. 2015).
65 Moreover, while both male and female reproductive functions are sensitive to exposure to ionizing
66 radiation, the effects on males have been studied less (Fuller, Lerebours et al. 2015). Regarding the
67 ICRP DCRLs, effects on crustaceans have been recorded from $0.42 \text{ mGy}\cdot\text{h}^{-1}$ with few results on

68 reproductive capacities and mortality (ICRP 2008). These data deal with (i) the hatching success of
69 the calanoid copepod *Diaptomus clavipes*, the fertility and fecundity of the females amphipod
70 *Gammarus duebeni*; (ii) the proportion of total deaths at molting after the chronic exposure of the
71 blue crab *Callinectes sapidus* at a dose rate of 70 mGy⁻¹ for 70 days; and (iii) the mortality (LD50/40,
72 corresponding to LD50 after 40 days) at 15 Gy for post-juvenile grass shrimp *Palaemonetes pugio*.
73 Other data were mainly obtained under acute conditions and taking into account radiation other
74 than gamma, e.g. X-rays (*Gammarus duebeni*).

75 Gammarids (*Gammarus* sp.) are amphipod crustaceans with sexual reproduction and often abundant
76 in aquatic ecosystems (Europe: *G. fossarum* and *G. pulex*, Asia: *G. nipponensis*...). They play an
77 important ecological role as detritivores, significantly contributing to the degradation of leaf litter in
78 watercourses and therefore to the nutrient cycle (Kelly, Dick et al. 2002). They are used as sentinel
79 organisms in ecotoxicology, and recognized for their sensitivity to the quality of the environment and
80 characterized in terms of their biological responses to numerous chemical compounds (metallic and
81 organic traces) (Kunz, Kienle et al. 2010; Chaumot, Geffard et al. 2015). In addition, some normalized
82 bioassays, such as feeding behavior or reprotoxic response measurements are available to assess the
83 response to chemical contaminants (Geffard, Xuereb et al. 2010; Coulaud, Geffard et al. 2011). Thus,
84 *G. fossarum* is a freshwater species used (i) to assess the ecotoxicity of chemical substances in
85 aquatic environments (caging in situ bioassays on rivers, effluents, etc.), or in laboratory (Geffard,
86 Xuereb et al. 2010; Lopes, Chaumot et al. 2020), (ii) as a tool for biomonitoring chemical status and
87 as a bioindicator for the ecological status of a wide variety of aquatic systems (standardized AFNOR
88 XP T90-721 (Babut, Ferrari et al. 2020), AFNOR XP T90-722-1/2/3 according to the effect criteria),
89 within the Water Framework Directive (WFD, EU 2013) (Alric, Geffard et al. 2019).

90 Gammarids have not been extensively documented in terms of IR, however they could offer a
91 relevant tool for biomonitoring radio-contamination after the characterization of radio-induced
92 biological effects. Thus, to increase our knowledge of radiation protection for a freshwater
93 crustacean with a sexual reproductive strategy, we undertook a dose-response study in controlled
94 laboratory conditions at five external gamma irradiation levels (0.0042, 0.047, 0.57, 5.01, 51mGy⁻¹)
95 and a control condition. This range of irradiation levels (dose rates) includes low levels (0.0042 and
96 0.047 mGy⁻¹) framing the threshold value of 10 µGy⁻¹ (Garnier-Laplace, Della-Vedova et al. 2010)
97 and level of 51 mGy⁻¹, close to those recorded in post-accident situations (e.g. immediately after the
98 Chernobyl nuclear accident (Garnier-Laplace, Geras'kin et al. 2013)). This study aims to assess
99 phenotypical responses after the continuous γ-irradiation of either males or females. In particular,
100 life history traits, such as survival, feeding behavior, molting and reproductive ability, in male or
101 female *Gammarus fossarum*, were assessed after exposure during a reproductive cycle (14 days).
102 Beyond that, since ionizing radiation is a well-characterized genotoxic stressor that can interact

103 directly with living matter, the knowledge of its ecotoxic profile from genotoxic effects to individual
104 adverse outcome, without interference from pollutant biokinetics, can be useful to understand
105 ecotoxicity of other chemical pollutants likely to exhibit genotoxicity.

106

107 MATERIAL AND METHODS

108 Organism sampling and maintenance

109 The gammarids came from the River Le Pollon, located in Saint-Maurice-de-Remets, France
110 (45°57'25.8''N 5°15'43.6''E), a source population characterized by very low pollutant concentration
111 levels (Alric, Geffard et al. 2019). They were collected using a hand-held net in March and June 2021.
112 Adult organisms were recovered through 2 and 2.5 mm sieves and then quickly transferred to the
113 laboratory. The organisms were acclimatized for a period of two weeks in 30 L tanks continuously
114 supplied with drilling groundwater (300 $\mu\text{S}/\text{cm}$) and constantly aerated. A 10/14 h light/dark
115 photoperiod was maintained, and the temperature was kept at 12 ± 1 °C. Organisms were fed *ad*
116 *libitum* with alder leaves (*Alnus glutinosa*), previously conditioned for at least four days in water.
117 Freeze-dried *Tubifex* worms were added as a dietary supplement twice a week.

118

119 Gammarid irradiation and experimental design

120 The gammarids were irradiated in incubators (Panasonic MIR 54 without light, at 15.5°C controlled
121 with Tinytag Aquatic 2[®] data loggers) for 14 days. The gammarids were placed in 500 mL glass
122 beakers filled with drilling water from our laboratory, previously tempered at 15.5°C and oxygenated
123 for 24 hours. This temperature, widely used for ecotoxicological experiments, was chosen as an
124 intermediate between the holding temperature and a temperature which accelerates the
125 reproductive cycle in order to perform the chronic exposure over the development period of
126 females, avoiding a too important cumulative dose. Water was renewed every two days and dead
127 organisms were removed. Beakers were placed according to a specific layout obtained based on
128 Monte Carlo simulations to achieve the required dose rate for each experimental unit. Two Radio
129 Photo Luminescent dosimeters (RPL, GD-301 type, Chiyoda Technol Corporation, Japan) were placed
130 on beakers (one at the front, one at the rear) in order to calculate the average dose rate in the
131 sample units, i.e. with the ratio between dose measured by dosimeters and the irradiated period.
132 Pieces of plastic mesh were placed in each beaker as shelters to avoid cannibalism.

133 *Gamma*-rays were emitted from external ¹³⁷Cs sources using the MICADO-Lab facility (*Moyen*
134 *d'Irradiation Chronique pour l'Acquisition de relations DOse-effet en Laboratoire*) (370 GBq
135 (Cadarache, FRANCE). Five dose rates and a control, 0, 0.005, 0.054, 0.57, 5.01 and 51.5 mGy⁻¹,
136 corresponding to six cumulative doses after 14 days (0, 1.7, 18.1, 191.7, 1 683, 17 312 mGy), were

137 tested. The control condition was kept at a distance in the same room. In addition, it was offset from
138 the source and separated with a concrete wall to maintain a low dose rate (130-140 nGyh⁻¹, reaching
139 0.03 mGy after 14 days).

140

141 **Biological parameter measurements**

142 The following parameters were chosen on the basis of proven protocols used for chemical pollutants
143 (Geffard, Xuereb et al. 2010; Coulaud, Geffard et al. 2011; Coulaud, Geffard et al. 2014), with regard
144 to the existing literature on the effects of ionizing radiation (Fuller, Lerebours et al. 2015) and the
145 known radiosensitivity of reproductive capacity (Garnier-Laplace, Della-Vedova et al. 2010).

146 **Survival:** gammarids were counted for survival rate assessment for each assay after 7 or 14 days of
147 irradiation.

148 **Feeding assay:** The feeding rate during irradiation was assessed according to the procedure
149 described in Coulaud et al (2011) (Coulaud, Geffard et al. 2011). Two series of experiments were
150 carried out in March and June 2021. Three replicates of 20 male gammarids of homogenous size (11
151 ± 0.7 mm) per condition were considered in the first series and four replicates of 10 synchronized
152 (i.e. at the same molting stage) female gammarids (10.3 ± 0.9 mm) per condition were considered in
153 the second series. After 7 days, leaf discs were collected and numerically scanned to estimate leaf
154 consumption. Feeding rate (FR) is expressed as a consumed surface per day per gammarid (mm² d⁻¹
155 organism⁻¹).

156 **The reproductive capacities of females:** As described by Geffard et al. (2010) (Geffard, Xuereb et al.
157 2010), the reproductive cycle of *Gammarus* females is synchronized with their molting cycle.
158 Synchronized females were selected at the start of their reproductive/molting cycle, between 0 and
159 48 hours after ecdysis (stage AB). Each molt stage is characterized by a specific marsupial embryonic
160 development stage and the size of developing follicles visible on the ovarian membrane. Six molt
161 stages were defined, A and B for postmolt, C1 and C2 for intermolt, and D1 and D2 for premolt. The
162 mean follicle surface is highly dependent on the molting stage, with the highest follicle growth
163 occurred during the C2 stage. In AB females, stage 1 embryos correspond to newly fertilized,
164 undifferentiated eggs. Then embryos are characterized by a comma-like shape (stage 2), which
165 appeared during the C1 molting stage. Stage 3 embryos appeared at the C2 molting stage and are
166 characterized by the presence of cephalothorax and segmented appendages. At stage 4, the
167 compound eye is fully visible and appendages are also fully developed. Stage 5 corresponds to newly
168 hatched juveniles.

169 Reproductive output was assessed by euthanizing 15 females for each condition at the end of
170 exposure. When reproductive output is assessed, females are expected to be in the middle of the
171 reproductive cycle, i.e. in stage C2 (Geffard et al (2010)).

172 The molting stage, the number of oocytes, and the number of embryos per female were evaluated as
173 described by Geffard et al (2010). The integumental morphogenesis of the third and fourth peritopod
174 pairs was observed by optical microscopy ($\times 200$) to identify the molting stage of the euthanized
175 females, as described by Geffard et al. (2010).

176 Embryos were manually recovered from the marsupium and counted under a binocular microscope.
177 The number of oocytes per female was counted by *in vivo* observation of the gonads under a
178 binocular microscope. Since the number of oocytes and embryos depends on female body size and
179 molting stage, this number was standardized by dividing the total count of oocytes and embryos by
180 female body size minus size at puberty (5 mm). Only females at molting stages C2 and D1 were
181 considered, as described in Geffard et al 2010.

182 Female gammarids were fed *ad libitum* with alder leaves. These measurements were repeated twice,
183 in March and June 2021, with a different batch of females each time.

184 ***Post-exposure reproductive assessment of males and females:*** After 14 days of irradiation, batches
185 of irradiated male and female gammarids were reciprocally paired with non-irradiated organisms and
186 were kept in the laboratory in control conditions for a new reproductive/molting cycle. Regarding the
187 batch of irradiated males, three replicates of five organisms were paired with the same number of
188 non-irradiated females, at the very end of the molting and reproduction cycle, just before ecdysis.
189 Concerning the batch of irradiated females, at the start of irradiation, female gammarids were
190 selected in the middle of their molting and reproductive cycle (i.e. in stage C1) so that the end of
191 irradiation coincided with the day before ecdysis (Chaumot, Coulaud et al. 2020; Lopes, Chaumot et
192 al. 2020). Thus, two replicates of ten organisms were paired with the same number of mature non-
193 irradiated males. During this post-exposure period, organisms were placed in separate cages, with
194 one cage per condition. The organisms were kept at 16°C, continuously supplied with drilled water
195 and constantly aerated. A few days after pairing, the cages were monitored to ensure that the
196 females laid eggs. Female gammarids that had not laid eggs after a few days were removed. The
197 embryos produced were counted seventeen days after ecdysis and egg laying by the females.

198

199 **Statistical analysis**

200 All statistical analyses were run using R software version 3.2.4. Control and irradiation or recovery
201 exposures were compared. Differences in survival, consumption rates, post-irradiation fertility and
202 the percentage of females without embryos between conditions were tested by a pairwise
203 comparison adjusted for multiple comparisons by Bonferroni corrections. The nonparametric test
204 (Kruskal-Wallis) was used to compare both fecundity and fertility between conditions. Proportion
205 tests were used to identify differences in molting rates versus the control conditions. The significance
206 level (alpha risk) was set at 0.05.

208 RESULTS AND DISCUSSION

209 - Survival assay

210 After 14 days of *gamma* irradiation, the gammarid survival rate remained high for both males and
211 females at all doses with mean survival systematically above 70% (**Table 1**). Our dose rate exposure
212 and maximum cumulative dose (17.3 Gy) results match all existing data for crustaceans after gamma
213 exposure (Fuller *et al*, 2015), meaning that no mortality is expected under 50 mGyh⁻¹ (~15 Gy). In
214 particular, data showed that 30d LD50 has been assessed at 15 Gy for grass shrimp, *Palaemonetes*
215 *pugio*. In addition, mortality is reached for dose rates as high as 212 mGyh⁻¹ for *Daphnia Pulex* when
216 irradiated after 385 days (~twelve “generations”, cumulative dose / generation ~ 152 Gy), or, 290
217 mGyh⁻¹ for *Callinectes sapidus* over a 70-day period (cumulative dose ~ 487 Gy).

218

219 - Feeding assay

220 No significant difference in feeding rate was observed between the control conditions and
221 gammarids irradiated to different doses, for both males and females (**Figure 1** – Pairwise
222 comparisons, Bonferroni correction, adjusted p-value>0.05).

223 Feeding, linked to general health status of organisms, is a well-known marker in ecotoxicology, and is
224 sensitive to different types of chemical contamination, indicating general toxicity in various pollution
225 contexts (agricultural, mining, urban, industrial) (Coulaud, Geffard *et al.* 2015). While this experiment
226 is usually carried out on males, we targeted females in order to obtain the levels of food
227 consumption in the irradiated females and take this data into account regarding female reproductive
228 output as described by Coulaud *et al.* (2015). Only synchronized females were selected in the first
229 stage of their reproductive cycle to avoid varying leaf consumption depending on the stage of their
230 reproductive cycle. Gamma radiation did not alter the feeding behavior of gammarids at the tested
231 doses during the exposure time regardless of the sex. This result is consistent with the absence of
232 effect on *Daphnia magna* filtration rate after chronic external irradiation (Gilbin, Alonzo *et al.* 2008)
233 or alpha contamination (Alonzo, Gilbin *et al.* 2008). However, this results on 14j does not presume of
234 the results on its detritivore capacities usually assessed on longer period.

235

236 - Molting cycle assessment in females

237 The proportions of females before or after the C2 stage of the reproductive cycle are shown in
238 **Table 2**. 15 females were used for each condition to determine their molting stage. For the two
239 series of experiments, after 14 days of exposure, around 70% of the control females were at molting
240 stage C2. At all doses, the proportions of females at stage C2 do not differ significantly (proportion
241 test, experiments 1 & 2; p-value >0.05). The molting cycle of females was not delayed by gamma

242 radiation exposure. Molting is a key physiological process for crustacean growth and reproduction
243 (Gouveia, Bonneton et al. 2018; Street, Eytcheson et al. 2019). This process is controlled by
244 hormones, and a number of chemicals, some of which act as endocrine disruptors, have been shown
245 to affect molting, even inducing increased mortality for the organisms in some cases, such as for
246 *Daphnia* (Song, Villeneuve et al. 2017; Chen, Gu et al. 2019; Wang, Yang et al. 2023). To our
247 knowledge, only one study has tested the effects of gamma radiation on the molting of crustaceans
248 (Han, Won et al. 2014). In this study, acute gamma radiation affected molting during the larval stage
249 in the copepod *Tigriopus japonicas*. The lack of observed effects in the adult gammarids investigated
250 in this study suggests that the susceptibility of the molting process to gamma ray radiation may
251 depend on the species and life stage and warrant further studies to explore these differences across
252 crustacean species.

253

254 - Reproductive capacity of irradiated females

255 **Figure 2** shows the numbers of oocytes and embryos (normalized per the female size) produced
256 during the irradiation period for the two series of experiments. The number of oocytes produced was
257 not significantly different whatever the dose rate; however, a non-significant trend to decrease in the
258 production of the number of oocytes seems to be observed at the highest dose (Kruskal Wallis test,
259 A1 p-value: 0.069, A2 p-value: 0.36). In addition, the number of embryos produced during irradiation
260 was not impacted at any dose (Kruskal Wallis test, B1 p-value: 0.72, B2 p-value: 0.45). This result is
261 consistent with an *in situ* study finding no evidence of impact on female *A. aquaticus* reproductive
262 parameters of current dose rates in aquatic systems at Chernobyl (< 27.1 $\mu\text{Gy/h}$) (Fuller, Ford et al.
263 2018). However, brood size is not fully integrative of reproductive impairment assessment since it
264 considers a number of embryos produced but not the viability of progeny that can also depend on
265 the quality of oocytes. Another study found that the brood size of the parthenogenetic *Daphnia*
266 *magna* was significantly reduced after chronic exposure to gamma rays (35mGy^{-1}). While the
267 authors did not consider whether it was due to inadequate oogenesis, embryogenesis or both
268 (Parisot, Bourdineaud et al. 2015), other paper described a decrease in ovulation rate in *C. elegans*
269 with no impact on hatching success after chronic irradiation exposure at the same dose rate
270 (Dufourcq-Sekatcheff et al 2021).

271 - Post-exposure reproductive assessment of males and females

272 As described by Geffard et al. 2010, oocytes produced during a molting cycle contribute to the
273 formation of the embryos in the next brood. In order to assess the impact of gamma radiation on the
274 quality of the gonads, we analyzed the ability of males and females to reproduce after 14 days of

275 irradiation. Batches of irradiated organisms were maintained in the laboratory during a new molting
276 cycle, without irradiation, and paired with non-irradiated organisms.

277 **Figure 3** shows the amounts of embryos produced (A) in non-irradiated females after mating with
278 irradiated males, (B) in irradiated females after mating with non-irradiated males as well as the
279 results of females with (C) and without (D) embryos from irradiated females paired with non-
280 irradiated males.

281 The fertility of the females was significantly impacted when the males had been irradiated at the
282 highest doses, with a threshold effect observed from 5 mGyh⁻¹ and a median embryo production of
283 0.2 embryos/female at the highest dose (Pairwise comparisons, Bonferroni correction, p<0.01). This
284 result could be due to unrepaired DNA damage in male gametes as previously observed by (Lacaze,
285 Geffard et al. 2011). In particular, reproduction defects in males have already been observed for
286 levels of DNA damage exceeding a minimal threshold suggesting that gammarid spermatozoa are
287 extremely sensitive to genotoxic insults and unable to repair this damage (Lacaze, Geffard et al.
288 2011). Only scarce data in the literature describes the effects on crustaceans after chronic exposure
289 to ionizing radiation, especially on males (Fuller, Lerebours et al. 2015) but our results are consistent
290 with the ones observed after exposure of male *E. marinus* to ³²P β-emitter at a lower dose rate (up to
291 0.4 mGyh⁻¹) (Fuller, Smith et al. 2019). In that paper, significant effects were observed on resultant
292 female fecundity at all dose rates and in the frequency of embryo abnormalities per female for males
293 exposed to 0.04 and 0.4 mGy.h⁻¹. *E. marinus* also exhibit a statistically significant reduction of sperm
294 quality with significant differences in DNA damage for the higher dose rate but no significant effects
295 recorded for sperm count. In the same paper no significant results were found on male *G. pulex* (for
296 which high mortality was observed in all groups including controls) which can be consistent with our
297 observed effects that seem starting to appear between 0.57 and 5 mGyh⁻¹.

298 A decrease in the fertility of females was also observed when they were irradiated during the
299 previous reproductive cycle (exposure between stages C1 and D2) and paired with non-irradiated
300 males. While high inter-individual variability was observed, mainly due to the high percentage of
301 females without embryos in the marsupium increasing rapidly in proportion to the exposure dose
302 rate (figure 3D), a decreasing trend was observed starting from the lowest dose (0.5 mGyh⁻¹) (figure
303 3C). However, even at the highest dose, some females managed to produce embryos, which is not
304 the case when the males were irradiated (only a tiny number of embryos were produced). This result
305 can be linked to the oocyte production during irradiation. At this step no significant results were
306 found on the number of oocytes (only a trend to decrease). On another invertebrate, ionizing
307 radiation have been shown to slow down oocyte production, probably due to apoptosis and cell cycle
308 arrest (Dufourcq Sekatcheff, Godon, et al. 2023). This hypothesis remains to be investigated in
309 freshwater crustaceans.

310 In terms of the differences in female fecundity observed after male or female irradiation, these
311 results are consistent with both the genotoxic properties of ionizing radiation (Pereira, Bourrachot et
312 al. 2011) and the conclusions of Lacaze, Geffard et al. 2011) for experiments on *Gammarids* with
313 genotoxicants, i.e. that spermatozoa were considerably more sensitive to DNA damage than oocytes.
314 This has already been identified in other organisms and partly attributed to a loss of apoptotic
315 capabilities during spermatogenesis (Aitken and De Iuliis 2007). For instance, in the soil invertebrate
316 *C. elegans* after irradiation, cell cycle arrest to enable DNA repair and increasing apoptosis occurred
317 in oocytes whereas spermatids were 40% less numerous in the same condition of irradiation, but
318 without involvement of apoptosis (Dufourcq-Sekatcheff et al 2021; Dufourcq Sekatcheff, Godon, et
319 al. 2023).

320 Other studies described in the literature are mainly performed in acute exposure conditions. For the
321 dipteran *Drosophila suzukii*, full female sterility was achieved at 75 Gy, while 99.67% male sterility
322 was observed at 200 Gy, suggesting that females are more sensitive than males for this species
323 (Krüger, Schlesener et al. 2018), while male and female sterility was observed at similar doses for
324 another dipteran, *Anastrepha fraterculus*, at 50 Gy for females and 70 Gy for males (Krüger, Ferreira
325 et al. 2021). Interestingly, sexual dimorphism is not systematically evaluated in arthropods as an
326 effect of chronic exposure to gamma radiation and may actually vary across species and type of
327 exposure.

328 - Radiological protection concerns

329 Our results on reproductive parameters showed that reproduction is the most radiosensitive
330 phenotypic endpoint in gammarids. Indeed, neither mortality nor short-term food consumption,
331 proxy of their general health status, are impacted in our controlled conditions of irradiation. This
332 work is consistent with many other studies in invertebrates describing radiation-induced
333 reprotoxicity as the most radiosensitive individual endpoint among the ones used to derive species
334 radiosensitivity curves i.e. mortality, morbidity, reprotoxicity (Garnier-Laplace, Della-Vedova et al.
335 2010; Beresford, Horemans et al. 2020; Shuryak 2020).

336 In addition, our results showed that radio-induced reprotoxicity is more severe for male than female
337 gammarids after chronic exposure. Here, we reported significant effects on male reproductive
338 capacity at 5 mGyh⁻¹ in the parental generation of a freshwater crustacean which is much lower than
339 for other crustaceans such as *Daphnia magna* (35 mGyh⁻¹) regarding a phenotypical endpoint
340 (Parisot, Bourdineaud et al. 2015). Excepted the data on male marine crustaceans *E. marinus* (Fuller,
341 Smith et al. 2019) most data on chronic exposure to gamma irradiation for other crustaceans only
342 demonstrated molecular damage at such exposure dose rates (Fuller, Lerebours et al. 2015) that

343 remain high dose rates only present after nuclear accidents. And, many data on freshwater
344 crustaceans come from studies on the (female) parthenogenetic species *Daphnia Magna*.
345 The dose rates found for the radio-induced phenotypical effects observed for the parent generation
346 exposed for 14 days are consistent with the ICRP DCRLs for crustaceans defined as between 0.4 and 4
347 mGy h⁻¹. However, this observed No-Effect Dose Rate is likely to be decreased if results observed
348 after multi-generational exposure on *Daphnids* (Parisot, Bourdineaud et al. 2015) can be
349 extrapolated for *Gammarids*.
350 Finally, these *Gammarus fossarum* data seem confirming the biological key events (KE)
351 “oogenesis/spermatogenesis impairment” in females/males, leading to the adverse outcome
352 “reproduction decrease” previously identified in an Adverse Outcome Pathway consensus (AOPc) set
353 up with the results of many taxonomic group (Tollefsen, Alonzo et al. 2022). Further analysis of sub-
354 individual endpoints will enable to decipher biological pathway involved in the observed effects and
355 to fuel the up-stream KE of this previous AOPc scheme. This can also enable to link together events at
356 different biological levels and get sub-individual key events likely to be sensitive at lower dose rates.
357 All of that requires further investigations but provides worthwhile prospects for ERA.

358

359 CONCLUSION

360 Our results showed that short-term food consumption in *Gammarids* is not impacted in our
361 conditions while reproduction is the most radiosensitive phenotypic endpoint of those tested. This is
362 of main interest since reproduction is a key ecological parameter as the ability of a species to
363 reproduce directly influences its population dynamics. In addition, our results suggest the greater
364 sensitivity of males, as previously observed in other species (Lacaze, Geffard et al. 2010;
365 Dufourcq-sekatcheff, Cuiné et al. 2021) but for which little data is available for crustacean taxa
366 (Fuller, Lerebours et al. 2015). This observation of effects for the first generation, after continuous
367 exposure over 14 days, also confirm the chance of observing earlier effects at lower dose levels if
368 irradiated for longer, over several generations, as in the freshwater ecosystems, since the response
369 of the population tends to change and worsen over generations (Parisot, Bourdineaud et al. 2015).
370 This ecotoxic profile at the individual biological level for ionizing radiation, that is a known well-
371 characterized genotoxic stressor, may be helpful to fuel a database of adverse outcomes likely to be
372 induced by other chemical pollutants with genotoxic properties. This is a first step in the
373 characterization of *Gammarus fossarum* facing ionizing radiations in order to fuel the existing AOPc
374 on reproductive impairments and before its further use.

375

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381

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496 **TABLES**

497

498 **Table 1** : Percentage of gammarids survival^a at different dose rates of *gamma*-rays after 14d of exposure – No
 499 significant differences between conditions were observed.

Dose rate in mGy.h ⁻¹	0	0.005	0.054	0.57	5.01	51.5
Total cumulated dose in mGy	0.0 3	1.70	18.1	191.7	1 683	17 312
Males						
14 d	n=3 78.3 (70 - 85)	n=3 81.7 (75 - 85)	n=2 90 (85 - 95)	n=2 92.5 (85 - 100)	n=3 83.3 (75 - 95)	n=3 90 (90 - 90)
Females						
14 d	n=4 75 (70 - 80)	n=4 80 (40 - 100)	n=4 90 (80 - 100)	n=4 72.5 (60 - 100)	n=4 87.5 (80 - 90)	n=4 77.5 (40 - 100)

^aSurvival is indicated by the mean and the min-max values

500

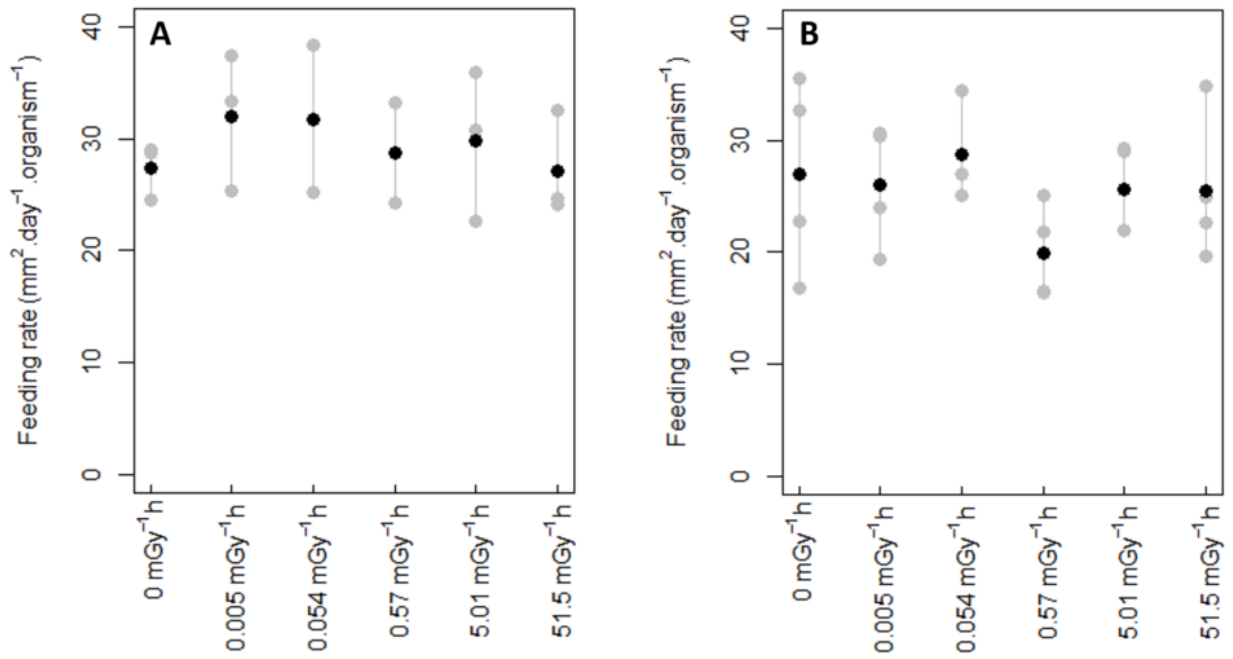
501 **Table 2** : Percentage of gammarid females in the different molting stages after 14 days of *gamma-rays*
 502 irradiation for the two series of experiments
 503

Percentage of Gammarids in the molt stage (%)						
Dose rate in mGy.h ⁻¹	0	0.005	0.054	0.57	5.01	51.5
Female - Experiment 1						
<i>n</i>	15	15	15	15	15	15
<C2	33.3	20.0	46.7	40.0	13.3	40.0
> C2	66.7	80.0	53.3	60.0	86.7	60.0
Female - Experiment 2						
<i>n</i>	15	15	15	15	15	15
<C2	26.7	46.7	20.0	26.7	20.0	20.0
> C2	73.3	53.3	80.0	73.3	80.0	80.0

504
 505
 506

507 **FIGURES**

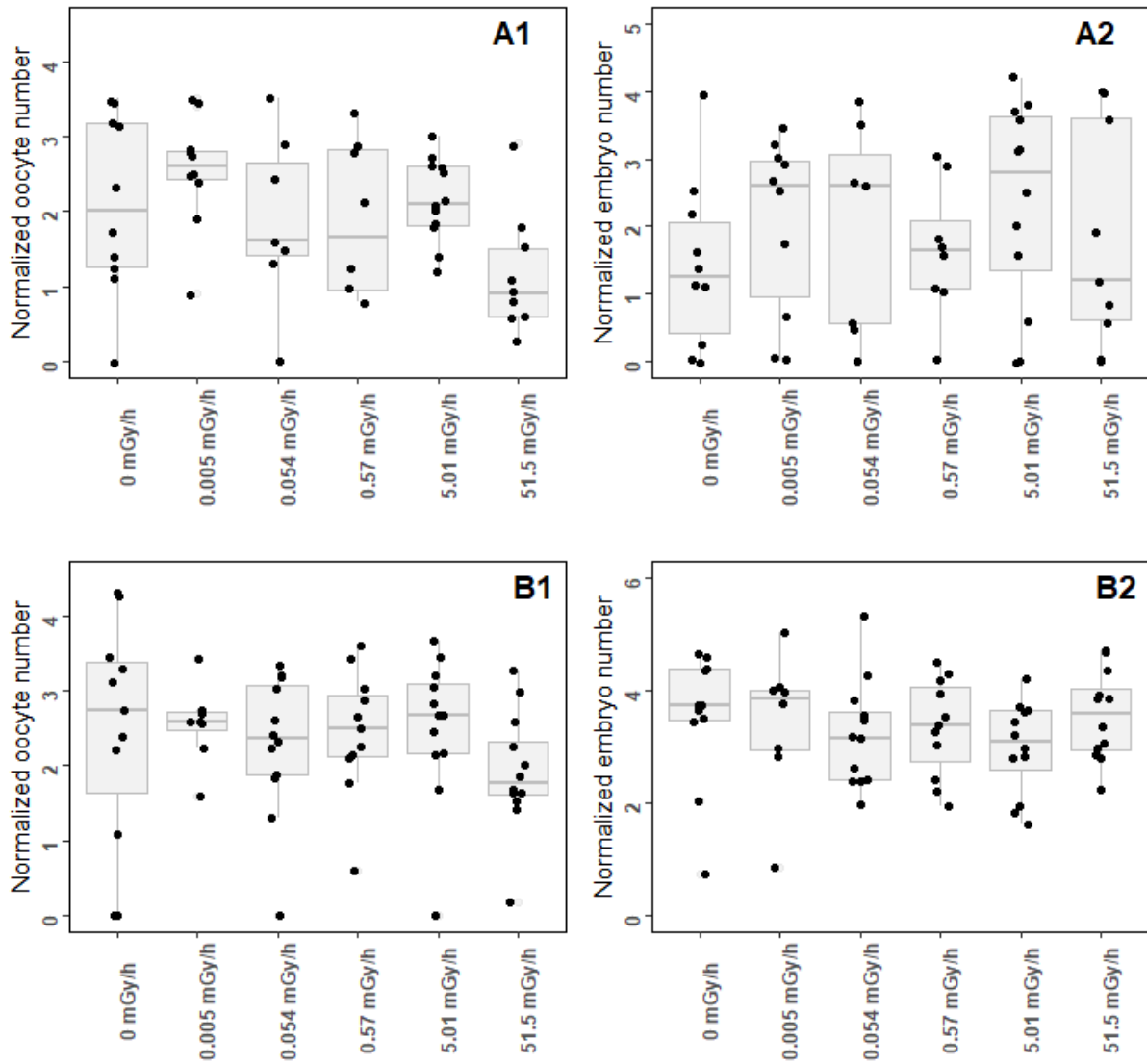
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509

510 **Figure 1** : Measurement of feeding rates (FR) at different doses of γ -rays after 7d of exposure (black
511 points: mean of experimental replicates; grey points: replicate value per dose) for (A) male gammarids (number
512 of replicates = 3 beakers of 20 males); and (B) molting synchronised female gammarids (number of replicates =
513 4 beakers of 10 females)

514



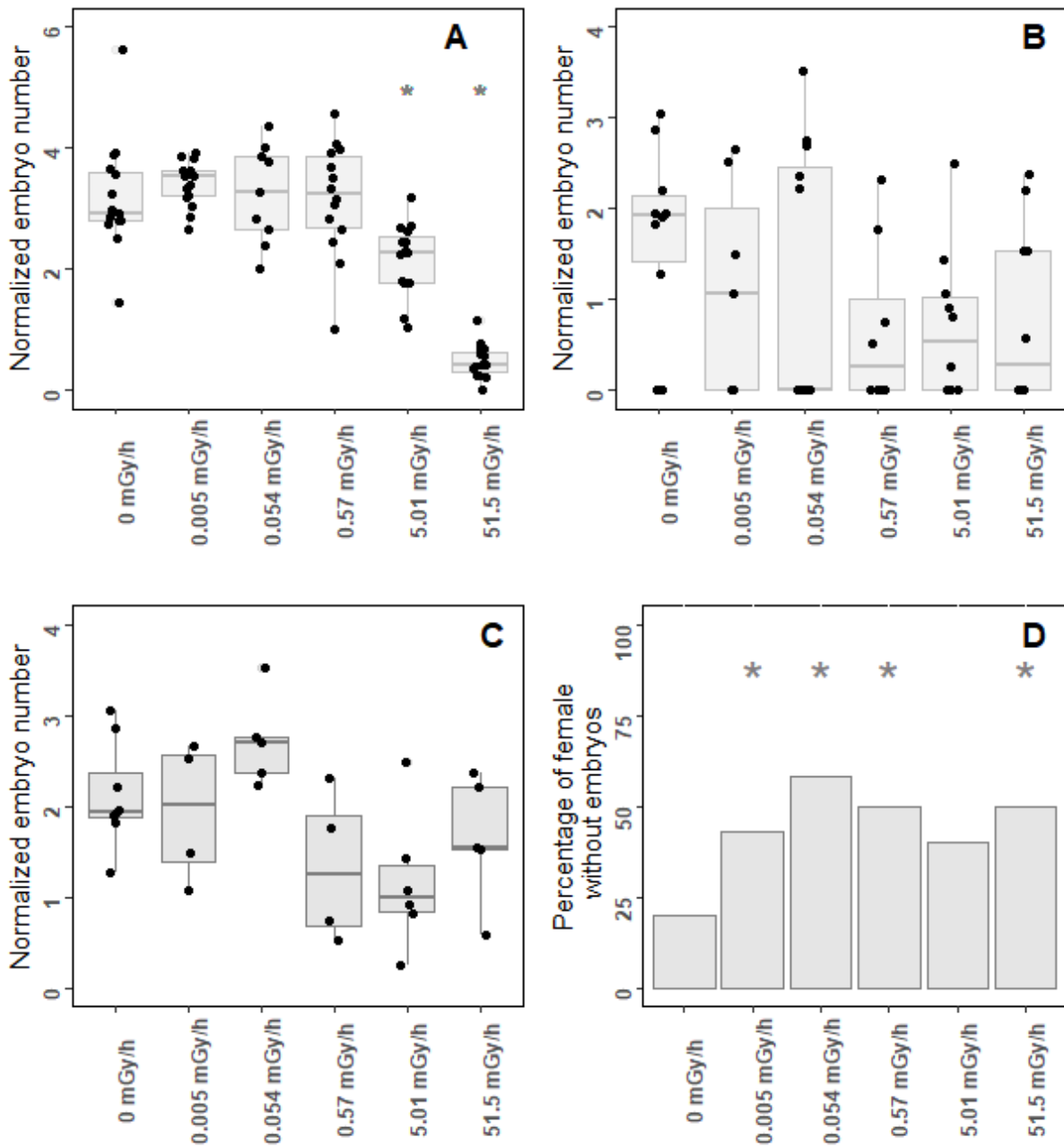
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516 **Figure 2** : Individual responses to 14-days *gamma*-rays exposure at different doses during two experiments.

517 (A1) and (A2) Oocyte production (size-normalized oocyte number per female). (B1) and (B2) Embryo production

518 (size-normalized embryo number per female). The median, first and third quartile are shown.

519



520

521 **Figure 3:** Effect of 14 days irradiation with gamma-rays at different doses on the production of embryos
 522 resulting from a new reproductive cycle without irradiation. (A) Results from irradiated males paired with non-
 523 irradiated females, (n=3 beakers of 5 irradiated males and 5 females). (B) Results from all irradiated females
 524 paired with non-irradiated males (n=2 beakers of 10 irradiated females and 10 males). The median, first and
 525 third quartile are shown. (C) Results from irradiated females with embryos paired with non-irradiated males.
 526 The median, first and third quartile are shown. (D) Percentage of females without embryos from irradiated
 527 females paired with non-irradiated males. *means significant difference compared to control (p.value < 0.05 –
 528 For 5.01 mGy/h, p.value = 0.0506)

529