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## Physiological effects of gamma irradiation in the honeybee, *Apis mellifera*

Beatrice Gagnaire, M Bonnet, S Tchamitchian, Isabelle Cavalie, Claire Della-Vedova, Nicolas Dubourg, Christelle Adam-Guillermin, JI Brunet, L Belzunces

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1                    **Physiological effects of gamma irradiation in the honeybee, *Apis mellifera***

2

3    **Authors**

4    Gagnaire B.\*<sup>1</sup>, Bonnet M.<sup>2</sup>, Tchamitchian S.<sup>2</sup>, Cavalié I.<sup>1</sup>, Della-Vedova C.<sup>3</sup>, Dubourg N.<sup>1</sup>, Adam-  
5    Guillermin C.<sup>1</sup>, Brunet J.-L.<sup>2</sup>, Belzunces L.P.\*<sup>2</sup>

6

7    **Affiliations**

8    <sup>1</sup> Institut de Radioprotection et de Sureté Nucléaire (IRSN), PSE-ENV/SRTE/LECO, Cadarache,  
9    Saint-Paul-lez-Durance 13115, France

10   <sup>2</sup> INRA, Institut National de la Recherche Agronomique, Laboratoire de Toxicologie  
11   Environnementale, UR 406 A&E, CS 40509, 84914 Avignon Cedex 9, France

12   <sup>3</sup> Institut de Radioprotection et de Sureté Nucléaire (IRSN), PSE-ENV/SRTE/LRTA, Cadarache,  
13   Saint-Paul-lez-Durance 13115, France

14

15   **\* Corresponding authors**

16   Béatrice GAGNAIRE  
17   IRSN  
18   PSE-ENV/SRTE/LECO  
19   Cadarache  
20   13115 Saint-Paul-lez-Durance, France  
21   Tel. +33 442199493  
22   Email [beatrice.gagnaire@irsn.fr](mailto:beatrice.gagnaire@irsn.fr)  
23

24   Luc P. BELZUNCES  
25   INRA  
26   Laboratoire de Toxicologie Environnementale  
27   UR 406 A&E  
28   CS 40509  
29   84914 Avignon Cedex 9, France  
30   Tel. +33 43272 2604  
31   Email [luc.belzunces@inra.fr](mailto:luc.belzunces@inra.fr)  
32

33 **Abstract**

34 Terrestrial ecosystems are exposed to various kinds of pollutants, including radionuclides. The  
35 honeybee, *Apis mellifera*, is commonly used in ecotoxicology as a model species for evaluating the  
36 effects of pollutants. In the present study, honeybees were irradiated right after birth for 14 days with  
37 gamma rays at dose rates ranging between  $4.38 \times 10^{-3}$  and 588 mGy/d. Biological tissues (head,  
38 intestine and abdomen) were sampled at D3, D10 and D14. Ten different physiological markers  
39 involved in nervous (acetylcholinesterase (AChE)), antioxidative (catalase (CAT), superoxide  
40 dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)), immune system  
41 (phenoloxidase (PO)) and metabolism (carboxylesterases (CaEs) and alkaline phosphatase (ALP))  
42 were measured. Univariate analyses were conducted to determine whether each individual biomarker  
43 response was positively or negatively correlated with the dose rate. Then, multivariate analyses were  
44 applied to investigate the relationships between all the biomarker responses. Although no mortality  
45 occurred during the experiment, several biomarkers varied significantly in relation to the dose rate.  
46 Globally, the biomarkers of antioxidant and immune systems decreased as the dose rate increased.  
47 Reversible effects on the indicator of the neural system were found. Concerning indicators of  
48 metabolism (carboxylesterases), variations occurred but no clear pattern was found. Taken altogether,  
49 these results help better understand the effects of ionizing radiation on bees by identifying relevant  
50 physiological markers of effects. These results could improve the assessment of the environmental risk  
51 due to ionizing radiation in terrestrial ecosystems.

52

53 **Keywords**

54 Honeybee, *Apis mellifera*; biomarkers; gamma rays; acetylcholinesterase; phenoloxidase; catalase;  
55 superoxide dismutase; carboxylesterases.

56

57

## 58 **Introduction**

59 Protecting the environment in the context of global change and sustainable management of resources  
60 and ecosystems is a major concern worldwide. Environmental pollution is a major problem for human,  
61 animal and plant populations (Colosio et al., 2005). Among the different pollutants, radioactive  
62 elements, such as uranium, can occur naturally. However, artificial radionuclides can also be released  
63 by human activities through normal functioning conditions of nuclear fuel cycle installations,  
64 controlled wastes from industrial and nuclear medicine activities, nuclear waste storage sites, deposits  
65 from nuclear tests or nuclear accidents, such as those that occurred at Chernobyl and Fukushima.  
66 These releases lead to a worldwide background of absorbed dose rate ranging from  $5 \times 10^{-4}$  to  $4 \times 10^{-3}$   
67 mGy/d in the environment, depending on the geographic zone (UNSCEAR, 1996). This radioactivity  
68 can increase in accidental contexts, with absorbed dose rates of 24 mGy/d in the case of the  
69 Fukushima accident (Adam-Guillermin et al., 2016).

70 The classical approach used for environmental protection takes into account difference in sensitivity of  
71 living organisms (Species Sensitivity Distribution). This approach has been developed for chemicals  
72 since the 1980s and more recently for radioactive elements (Garnier-Laplace et al., 2006). Recent  
73 studies recommended a generic screening value of 0.24 mGy/d to protect aquatic ecosystems from  
74 chronic external gamma irradiation (Garnier-Laplace et al., 2010). However, knowledge on potential  
75 effects of radioactive elements at doses above this threshold value in non-aquatic ecosystems are  
76 poorly developed.

77 Ecotoxicological properties of ionizing radiation have not been extensively studied for nonhuman  
78 species, particularly for terrestrial invertebrates such as bees. Information regarding exposure to  
79 ionizing radiation in bees is limited to bioaccumulation data (Fresquez et al., 1997; Haarmann, 1997,  
80 1998a, b; Hakonson and Bostick, 1976). Further, information on mechanisms of toxicity, early and/or  
81 sublethal effects of exposure to ionizing radiation are scarce, despite the importance of bees for  
82 ecosystem sustainability. However, some field studies have revealed that populations of bumble-bees,  
83 spiders, grasshoppers and dragonflies decreased in highly radioactive areas of the Chernobyl

84 Exclusion Zone (Moller and Mousseau, 2009), whereas no significant declines of these groups were  
85 found in the zone around the Fukushima accident, at least during the first summer following the  
86 disaster (Mousseau and Møller, 2014). In this context, a better knowledge of mechanisms underlying  
87 these effects, at environmentally relevant doses, is therefore needed to predict the possible  
88 consequences of the exposure to ionizing radiation on the ecosystems.

89 One of the first impacts of a pollutant occurs at the cellular level (Baynes and Dominiczak, 2019;  
90 Krzystyniak et al., 1995). Pollutants can directly or indirectly affect major physiological systems,  
91 including the immune system, general metabolism, the detoxication system and neural activity.  
92 Pollutants may also elicit oxidative stress that damage cells and tissues, thereby eventually impairing  
93 these physiological systems. The alteration of one or several of these biological functions is likely to  
94 alter homeostasis and adaptability of the organisms to their environment, thus impairing growth,  
95 reproduction and survival. However, the effects at higher biological organization levels are always  
96 preceded by early modifications in biological processes. Such subtle modifications allow investigation  
97 opportunity to measure biomarkers of effects that can be considered physiological tools for assessing  
98 organism health, like in medical analysis (Baynes and Dominiczak, 2019). Hence, developing suitable  
99 diagnostic tools appears to be critical in the context of ecotoxicological risk assessment (Sanchez et  
100 al., 2012; Sanchez and Porcher, 2009).

101 In this context, we propose using the honeybee *Apis mellifera* L., 1758 (Hymenoptera: Apidae), as a  
102 model to study the effects of ionizing radiation on terrestrial organisms. Honeybees are insects of  
103 economic, agro-environmental and scientific importance. At the economic level, honeybees allow an  
104 important source of incomes for a whole agricultural branch, beekeeping, due to the production hive  
105 products presenting an important added value (Celli and Maccagnani, 2003). At the agro-  
106 environmental level, honeybees are an important plant pollinator and thus contribute to increase the  
107 quantity and the quality of crops (Gallai et al., 2009). They also increase plant biodiversity (Brown  
108 and Paxton, 2009). While foraging, honeybees can explore several kilometers from the hive to collect  
109 resources such as nectar, pollen, resins, and water, any of which may be in contact with different  
110 pollutants (Chauzat et al., 2009). Therefore, the honeybee is considered a bioindicator of high

111 sensibility for environmental quality (Thompson and Maus, 2007). In pesticide registration, the  
112 honeybee is also a model species for protected species in European Community countries and is  
113 recommended by OECD for normalized procedures to test the toxicity of pesticides (OECD).  
114 Scientifically, the honeybee is suitable for studying cognitive functions (Srinivasan, 2010). Bees are  
115 also suffering from an important worldwide decline, in which causes like climate change, loss of  
116 habitats, exposure to pollutants and infections by pathogens are suspected (Needleman et al., 2018;  
117 Rhodes, 2018). Finally, the honeybee is one of the Reference Animals and Plants (RAP) model of the  
118 International Commission for Radiological Protection, which reinforces its interest as a model species  
119 for studying the effects of ionizing radiation (ICRP, 2008). Moreover, honeybees have already been  
120 used to detect radionuclides, after the Chernobyl accident, and also for other industrial accidents  
121 (Porrini, 2008). Finally, several biomarkers have been developed for the honeybee, and some may  
122 potentially be used for assessing environmental quality (Badiou-Bénéteau et al., 2012b; Badiou and  
123 Belzunces, 2008; Hyne and Maher, 2003; Vasseur and Cossu-Leguille, 2003). These biomarkers,  
124 measured in the head, midgut and abdomen, can provide information on the integrity of the nervous  
125 system (acetylcholinesterase (AChE)), antioxidative defenses (catalase (CAT), superoxide dismutase  
126 (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)), immune system  
127 (phenoloxidase (PO)) and metabolism (carboxylesterases (CaEs) and alkaline phosphatase (ALP)). All  
128 of these biomarkers were validated after exposure to pesticides (Badiou-Bénéteau et al., 2013a;  
129 Badiou-Bénéteau et al., 2012b; Carvalho et al., 2013b).

130 According to the ICRP, honeybee mortality is suspected to occur at dose rates higher than  $1 \times 10^3$   
131 mGy/d and reproductive success can possibly be reduced for dose rates of  $1 \times 10^2$ - $1 \times 10^3$  mGy/d (ICRP,  
132 2008). The Dose Reference Consideration Levels (DCRL) for bees, for which deleterious effects are  
133 expected, are between  $1 \times 10$  and  $1 \times 10^2$  mGy/d (ICRP, 2008). Few data are available for lower dose  
134 rates. In this context, we investigated the effects of ionizing radiation on physiological markers of *A.*  
135 *mellifera* during a 14-day experiment of continuous irradiation to gamma rays ( $^{137}\text{Cs}$ ) at dose rates  
136 ranging from  $4.38 \times 10^{-3}$  (controls) to 588 mGy/d. After 3, 10 and 14 days, animals were sampled to  
137 measure the battery of physiological markers indicated above. Our main objective was to improve our

138 knowledge on effects of ionizing radiation on bee physiology, and on the mechanisms by which these  
139 effects are induced after a chronic exposure (i.e., exposure duration significant towards the organism  
140 lifespan) at low dose rate (subtoxic ecotoxicity). The results will help us for a better understanding of  
141 the impacts of ionizing radiation on invertebrate key species for terrestrial ecosystems like bees.

142

## 143 **Material and Methods**

### 144 *Chemicals*

145 All chemicals were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). The chemicals  
146 included: antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monobasic and dibasic  
147 sodium phosphate, sodium chloride (NaCl), triton X-100, acetylthiocholine iodide, 5,5'-dithio-bis(2-  
148 nitrobenzoic acid) (DTNB),  $\alpha$ - and  $\beta$ -naphthyl acetate ( $\alpha$ -NA or  $\beta$ -NA), *p*-nitrophenyl acetate (*p*-  
149 NPA), 1,5-bis (4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51), fast garnet  
150 GBC, sodium dodecyl sulfate (SDS), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), monobasic potassium phosphate,  
151 ethylenediaminetetraacetic acid (EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), reduced L-glutathione  
152 (GSH), oxidized glutathione (GSSG), acetonitrile, acetone, NADPH,  
153 tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), magnesium chloride (MgCl<sub>2</sub>), *p*-  
154 nitrophenyl phosphate (*p*-NPP), *tert*-butyl hydroperoxide (TBHP), 3,4-Dihydroxy-L-phenylalanine (L-  
155 DOPA) and bovine serum albumin.

156

### 157 *Honeybees*

158 Honeybees were reared at the experimental apiary of the Institut National de la Recherche  
159 Agronomique (INRA), Research Unit 406 *Abeilles & Environnement* (Bees & Environment),  
160 Avignon, France. The presence of a queen was checked, and the health status of the honeybees was  
161 continuously and carefully monitored. Workers were collected from the honey super compartment of  
162 the beehive, transferred to IRSN laboratories, put in cages (8 cm x 5 cm x 4 cm, 30 bees per cage), fed

163 ad libitum with candy paste and water. The insects were kept at  $32 \pm 2$  °C and  $60 \pm 10\%$  relative  
164 humidity.

165

#### 166 *Exposure to gamma rays*

167 Honeybees were exposed for 14 days to gamma rays emitted by a liquid  $^{137}\text{Cs}$  source in a polystyrene  
168 tube (20 MBq in HCl 0.1 M) or a solid  $^{137}\text{Cs}$  line source (1.85 GBq). Dose rates received by the bees  
169 in cages were characterized using RPL glass dosimeter measurements (Chiyoada Technologies, Japan)  
170 at  $4.38 \times 10^{-3}$  (controls), 0.336, 0.936, 3.36, 9.36, 33, 92.4, 210 and 588 mGy/d.

171 Every day, dead honeybees were removed from the cages and daily food consumption was measured  
172 and expressed as percentages of food consumption during the first day in controls. Living honeybees  
173 were randomly selected and removed at D0, D3, D10 and D14, and tissues were sampled and  
174 immediately frozen at  $-80^\circ\text{C}$  until biomarker analysis.

175

#### 176 *Tissue extracts*

177 To prevent any animal suffering, all tissues were removed from bees previously anesthetized and then  
178 decapitated. Honeybee heads were obtained by cutting from the body with a scalpel. Then, midguts  
179 were obtained by pulling the stingers from the honeybees. Abdomens correspond to abdomens devoid  
180 of intestinal tract. Tissues samples (pools of tissues from 5 bees) were placed in a 2-mL microfuge  
181 tubes. In order to provide enough material for analyses and to limit inter-individual variability, seven  
182 pools were made for controls and bees exposed at 210 and 588 mGy/d; four pools were made for the  
183 other conditions. The extraction buffer was added to make a 10% (w/v) tissue extract. The extraction  
184 buffer consisted of 1% (w/v) Triton X-100, 10 mM sodium chloride and 40 mM sodium phosphate at  
185 pH 7.4, and contained protease inhibitors (2 mg/mL antipain, leupeptin, and pepstatin A, 25 units/mL  
186 aprotinin, and 0.1 mg/mL soybean trypsin inhibitor) (Belzunces et al., 1988). The tissues were grinded  
187 in the extraction medium with a Qiagen® Tissue Lyser II (30 Hz, three periods of 30 sec, at 30 sec



188 intervals). The tissue extracts were centrifuged for 20 min at 16000 g, and the supernatants recovered  
189 for biochemical analyses was immediately used for marker analysis and then stored at -80 °C for  
190 protein content analyses. All extraction procedures were conducted at 4°C.

191

## 192 *Enzyme assays*

193 Enzyme assays were performed on microplates with UV-Visible Biotek Synergy HT  
194 spectrophotometer at 25°C in a final reaction volume of 200 µL. The activity of each sample was  
195 determined in triplicate. Protein concentration was quantified according to Markwell et al. (1978)  
196 using bovine serum albumin as a standard.

197 AChE was assayed at 412 nm in a medium containing 0.3 mM acetylcholine iodide, 1.5 mM DTNB,  
198 and 100 mM sodium phosphate at pH 7.0, according the method of Ellman et al. (1961) modified by  
199 Belzunces et al. (1988). Three CaEs were monitored: CaE1, CaE2, and CaE3 classified according to  
200 their substrate specificity corresponding to the hydrolysis of  $\alpha$ -naphthyl acetate ( $\alpha$ -NA),  $\beta$ -naphthyl  
201 acetate ( $\beta$ -NA) and *p*-nitrophenyl acetate (*p*-NA), respectively (Gomori, 1953). The crude tissue  
202 extract was incubated in a medium containing  $1 \times 10^{-4}$  M of BW284C51 as an AChE inhibitor and 100  
203 mM sodium phosphate, at pH 7.4, for 20 min at 25°C in the darkness. After incubation, the appropriate  
204 substrate ( $\alpha$ -NA,  $\beta$ -NA or *p*-NA) was added to a final concentration of 0.4 mM. For CaE1 and CaE2,  
205 the enzyme reaction was performed for 3 min and stopped with 1.5% SDS and 0.4 mg/mL fast garnet  
206 GBC. The reaction products were measured at 568 nm for  $\alpha$ -NA (CaE1) and 515 nm for  $\beta$ -NA (CaE2).  
207 For CaE3, the reaction was continuously monitored at 410 nm. Alkaline phosphatase (ALP) was  
208 monitored continuously at 410 nm in a medium containing 20 mM MgCl<sub>2</sub>, 2 mM *p*-NPP and 100 mM  
209 Tris-HCl at pH 8.5 (Bounias et al., 1996). Glutathione-*S*-transferase (GST) was measured at 340 nm in  
210 a medium containing 1 mM EDTA, 2.5 mM GSH, 1 mM CDNB and 100 mM sodium phosphate at pH  
211 7.4 (Habig et al., 1974). Catalase (CAT) was measured at 240 nm according to the procedure  
212 described by Beers and Sizer (Beers Jr and Sizer, 1952) in a medium containing 10 mM H<sub>2</sub>O<sub>2</sub> and 100  
213 mM phosphate at pH 7.0. SOD activity was measured at 560 nm in a reaction medium containing 0.1

214 mM EDTA, 0.1 mM xanthine, 0.025 mM nitroblue tetrazolium (NBT), 8.33 mU/mL xanthine oxidase  
215 and 50 mM sodium phosphate/carbonate at pH 7.8. GPx was measured at 340 nm in a medium  
216 containing 1 mM EDTA, 0.2 mM TBHP, 0.85 mM GSSG, 0.16 mM NADPH, 0.25 U/mL glutathione  
217 reductase and 50 mM Na/K phosphate at pH 7.4. Phenoloxidase (PO) was measured at 490 nm in a  
218 medium containing 20 mM NaCl, 2 mM L-DOPA and 10 mM sodium phosphate at pH 7.2.

219

## 220 *Data analysis*

221 Differences of mortality and food uptake were evaluated using Kruskal-Wallis test or ANOVAs on the  
222 STATISTICA Software version 12 (StatSoft, Inc., Tulsa, OK, USA), with significance judged at  
223  $p < 0.05$ .

224 Assessment of the impact of dose rate on each physiological marker response was performed at three  
225 observation times (D3, D10 and D14) using simple linear regression model, when possible. Response  
226 of measurement replicates were averaged before statistical analyses. Dose rate was transformed  
227 ( $\log_{10}$ ) because dose rate was highly spread (ratio max/min =  $1.2 \times 10^6$ ) and also in order to increase  
228 the linearity of biomarkers response. Since the assumption of linear relationship did not appear always  
229 obvious, fits of polynomials models with 1 (linear), 2 (quadratic), 3 (cubic) and 4 (quartic) degrees  
230 were compared, to help make a decision. Fits were compared in a stepwise backward approach using F  
231 tests and adjusting  $p$ -values for multiple comparisons. When linearity was accepted, residuals  
232 normality assumption was tested using Shapiro-Wilk test. When normality was not accepted, a log  
233 transformation, or a square root transformation, or a Box-Cox transformation was applied on the  
234 responses of physiological markers. Residuals homogeneity assumption was assessed visually using  
235 fitted vs standardized residuals plot. When it was not satisfied, a variance structure was added to the  
236 linear model. Finally, relationships between all physiological marker response and dose rate increase  
237 were studied by Principal Component Analysis and by a hierarchical clustering of variables approach,  
238 at the three different sampling times.

239 Statistical analysis were performed using R software version 3.3.2 (R\_Core\_Team, 2017), and  
240 RStudio environment version 0.99.484 (RStudio\_Team, 2015). When structure variance was needed,  
241 linear models were fitted using *gls* function of *nlme* package. *P*-values relative to comparisons of  
242 polynomial model fits were done using the single step approach of *multcomp* package. Level of  
243 significance was fixed at 5%. Principal components analyses were performed using the *FactoMineR*  
244 package. Hierarchical clustering of variables was done using the *ClustOfVar* package.

245

## 246 **Results**

### 247 *Mortality and feeding*

248 No difference of mortality was detected between controls and bees exposed at the different dose rates  
249 (Kruskal-Wallis test,  $p=0.214$ ). The mortality rates ranged between 0.6 and 8.6% (data not shown).

250 Similarly, no differences in the daily food intakes were shown between the different groups of  
251 exposure for the whole duration of the experiment (*t*-test or Kruskal-Wallis tests,  $p>0.05$ ). The daily  
252 food intake ranged between 6.8 and 12.1% of food consumption during the whole experiment (data  
253 not shown).

254

### 255 *Relationship between physiological marker levels and dose rate*

256 At D3, the activity of several physiological markers showed a significant positive linear relationship  
257 with the dose rate: that was the case for head CaE1 and CaE3, intestinal CAT and abdominal SOD  
258 (Table 1, Figure S1). However, intestinal GST presented a significant negative linear relationship with  
259 the dose rate (Table 1, Figure S1). Head CaE2 and GPx, intestinal CaE3 and abdominal CAT, GST  
260 and PO did not have linear significant relationships with the dose rate (data not shown). Other  
261 physiological markers presented complex and non-monotonic significant relationships with the dose  
262 rate. A two-order polynomial relationship was found for intestinal CaE1, with a decrease of activity  
263 for the lowest dose rates tested, and an increase of activity for dose rates higher than 10 mGy/d (Fig.

264 1A). Some physiological markers showed a three-order polynomial relationship with the dose rate:  
265 head AChE increased at low dose rates and decreased at dose rates higher than 1 mGy/d, then  
266 increased again for dose rates higher than 100 mGy/d (Fig. 1B); intestinal ALP and SOD decreased at  
267 low dose rates and increased at dose rates higher than 0.1 mGy/d (more pronounced for SOD) (Fig.  
268 1C,D). Finally, intestinal CaE2 and head CAT showed a four-order polynomial relationship with the  
269 dose rate, with a decrease at low dose rates and an increase at dose rates higher than 0.1 mGy/d, then  
270 another decrease for dose rates higher than 10 mGy/d (Fig. 1E,F).

271 At D10, intestinal CAT and GST presented a significant positive linear relationship with the dose rate,  
272 whereas head CaE1 and CaE2, intestinal CaE3 and head CAT were negatively correlated (Table 1,  
273 Figure S2). Non-significant linear relationships with the dose rate were found for head AChE,  
274 intestinal CaE1, CaE2, SOD and ALP and abdominal CAT, SOD and PO (data not shown). Other  
275 biomarkers exhibited significant correlations with the dose rate in a non-linear mode. Head CaE3  
276 presented a two-order non-monotonic polynomial relationship, with a decrease of activity for the  
277 lowest dose rates tested, and an increase of activity for dose rates higher than 1 mGy/d (Fig. 2A). Head  
278 GPx presented a three-order complex polynomial relationship with dose rate, with a decrease at low  
279 dose rates, an increase at dose rates higher than 0.1 mGy/d and another decrease for dose rates higher  
280 than 100 mGy/d (Fig. 2B).

281 At D14, intestinal CaE3 and head GP showed significant positive linear relationships with the dose  
282 rate (Table 1, Figure S3). Non-significant linear relationships with the dose rate were found for head  
283 AChE, intestinal CAT, GST and ALP, and abdominal GST (data not shown). Other biomarkers  
284 presented complex and non-monotonic significant relationships with the dose rate. Intestinal CaE1 and  
285 CaE2, and abdominal CAT and SOD showed a three-order polynomial relationship with dose rate,  
286 with a decrease of activity for the lowest dose rates tested, an increase of activity for dose rates higher  
287 than 1 mGy/d and another decrease for dose rates higher than 100 mGy/d (Fig. 3A,B,D,E). The same  
288 relationship was found for intestinal SOD but with only a slight decrease for the lowest dose rates  
289 tested and an increase of activity for dose rates higher than 0.1 mGy/d (Fig. 3C). Finally, several  
290 physiological markers were related to the dose rate with a four-order polynomial relationship. Head

291 CaE1, 2 and 3 increased for the lowest dose rates tested, decreased for dose rates higher than 0.1  
292 mGy/d, increased again for dose rates higher than 1 mGy/d and decreased again for dose rates higher  
293 than 100 mGy/d (Fig. 4A,B,C). Head CAT and abdominal PO decreased for the lowest dose rates  
294 tested, increased for dose rates higher than 0.1 mGy/d, and decreased again for dose rates higher than  
295 10 mGy/d (Fig. 4D,E). Only CAT increased again for dose rates higher than 100 mGy/d (Fig. 4D).

296

### 297 *Multivariate analyses*

298 The relationships between all of the biochemical biomarkers were analysed with PCA performed on  
299 the whole set of data for each sampling time (Fig. 5). For D3, the two first axes explained 40% of  
300 inertia. The first axis was explained by the three CaE and CAT in the intestine. To a lesser degree, the  
301 first axis was also explained by all head CaE. The second axis was explained by high values of  
302 intestinal and abdominal GST and abdominal and head CAT. Negative values of second axis were  
303 related to high values of intestinal SOD and ALP. Dose rate was poorly related to both axes (Fig. 5A).  
304 For D10, the two first axes explained 41% of inertia. The contribution of intestinal and head CaE on  
305 the first axis was similar to D3. Intestinal CAT was less related to the first axis, but intestinal SOD  
306 was more strongly related to the first axis. The second axis was very well explained by abdominal  
307 SOD, CAT and GST, which were very correlated each other, and also in a lower manner by abdominal  
308 PO and head GPx. As for D3, dose rate was poorly related to both axes (Fig. 5B). For D14, the two  
309 first axes explained 43% of inertia. The contribution of biomarkers to axes was different compared to  
310 D3 and D10. The first axis was still explained mainly by abdominal biomarkers (SOD, CAT and PO),  
311 while the second axis explained primarily by head biomarkers (CaE1, CaE2, CaE3). However, at D14,  
312 intestinal biomarkers were related to both axes. Moreover, abdominal PO and intestinal SOD were  
313 negatively correlated. Dose rate was associated with low values of the abdominal biomarkers  
314 contributing to first axis (Fig. 5B). For all sampling times, the three CaE presented high correlations  
315 between them in both organs.

316 A cluster analysis was also performed for each sampling time (Fig. 6). At D3, five clusters were  
317 relevant: the first cluster with the three head CaEs, the second cluster with the three intestinal CaEs  
318 and CAT, the third cluster with head AChE and CAT and intestinal GST, the fourth cluster with  
319 abdominal CAT, SOD and GST and the fifth cluster with head GPx, intestinal SOD and ALP and  
320 abdominal PO (Fig. 6A). At D10, five clusters were also relevant: the first cluster with all abdominal  
321 markers, the second cluster with head AChE, CAT, GPx, and the third cluster with the three head CaE  
322 and the intestinal GST. The two last clusters were composed of intestinal physiological markers, with  
323 all CaE for the fourth cluster, and CAT, ALP and SOD for the fifth one (Fig. 6B). At D14, five  
324 clusters emerged: the two first clusters were composed of intestinal markers, with CAT and ALP, and  
325 the three CaE for the first and second clusters, respectively. The third cluster was composed of head  
326 AChE and intestinal GST. The fourth cluster was composed of the intestinal SOD and the three head  
327 CaE. The fifth cluster grouped all abdominal markers (GST, PO, CAT and SOD) and head markers  
328 (CAT and GPx) (Fig. 6C). Globally, the cluster analysis showed like the PCA that the three CaE in  
329 head and intestine are highly correlated with each other. A cluster of abdominal biomarkers was also  
330 found for all sampling times, showing that the biomarker levels in abdomen evolve in a similar way.  
331 Contrary to PCA, the cluster analysis did not reveal that oxidative stress biomarkers are always  
332 related.

333

## 334 **Discussion**

335 The aim of the present study was to understand the effects of gamma irradiation on honeybees and to  
336 identify the mechanisms underlying the observed effects by measuring a battery of biomarkers  
337 involved in several physiological functions of bees.

338 Few studies have used the honeybee as a bioindicator in the context of radiation exposure (Badiou-  
339 Bénéteau et al., 2012b). Toxicological studies focused on honeybee physiological markers were  
340 initiated by Metcalf and March (1949). Later, Gilbert and Wilkinson (1974) and Yu et al. (1994)  
341 showed that carboxylesterases (CaEs), glutathione-*S*-transferase (GST), DDT-dehydrochlorinase, and

342 microsomal oxidases can be modulated by pesticides. Two decades were necessary for the use of  
343 honeybee acetylcholinesterase (AChE) as a biomarker to assess the impact of organophosphates and  
344 carbamates (Attencia et al., 2005; Stefanidou et al., 1998) and thereafter the use of other biomarkers to  
345 characterize and exposure to pesticides (Badiou and Belzunces, 2008; Hashimoto et al., 2003; Rabea et  
346 al., 2010). The honeybee matches well the definition of a bioindicator (Lagadic et al., 1997) because it  
347 is an abundant species in which effects of ionizing radiation can be observed, even at low dose rates,  
348 in individuals with a relatively short lifespan, the workers, and in an individual exhibiting a long  
349 lifespan, the queen.

350 The LD<sub>50</sub> of gamma radiations on large insects vary from 20 to 3000 Gy, with sub-adult stages being  
351 more sensitive (LD50 values of 1 to 2 Gy) (ICRP, 2008). In our study, bees received a maximum total  
352 dose rate of 14 Gy. Ionizing radiation induced physiological modifications on biomarkers in all of  
353 honeybee biological compartments considered. Such a distribution profile of effects showed that the  
354 response of honeybees to ionizing radiation is rather systemic and that the effects are not particularly  
355 concentrated in a given tissue that could be more susceptible to radiation. The physiological  
356 disruptions not only affected metabolic enzymes (CaE1, 2, 3) but also enzymes involved in the  
357 antioxidative defense system (CAT, SOD, GPx). This results shows that an oxidative stress, elicited by  
358 ionizing radiation, may also occur in the honeybee, even at low doses (Tharmalingam et al., 2017).

359 The profiles of the dose-response relationships of the different physiological markers are multiple and  
360 depended greatly on the marker considered, the biological compartment and the length of the period  
361 during which bees were exposed to ionizing radiation. The simplest dose-effect relationship presented  
362 a positive or negative linear profile. Slight variations (hyperbolic, gamma, Hill, Weibull etc.) of these  
363 profiles might be possible, but modelling failed to detect them and linear fitting remained the best  
364 model that accounts for a significant correlation between the biological effects and the dose rates. For  
365 the other dose-response relationships, complex non-monotonic profiles were observed. The simplest  
366 complex profile was the U-shaped biphasic dose-response relationships. This profile generally reflects  
367 either hormesis phenomenon, that may include overcompensation (Agathokleous, 2018), or  
368 compensation by feedback controls and induction followed by saturation of defense systems (Zhang et

369 al., 2015). Whatever the effects observed, such a non-monotonic profile is not surprising because all  
370 biological systems are regulated by positive and negative mechanisms of control, which make that  
371 stressors, such as pesticides, may also present non-monotonic dose-response relationships (Baines et  
372 al., 2017; Charpentier et al., 2014; Suchail et al., 2000). In addition, an adaptive response may occur  
373 and may vary with the dose rate of ionizing radiation (Wolff, 1998). Such adaptive mechanisms that  
374 may modulate the biological response to ionizing radiation have been known for more than 30 years  
375 (Shadley et al., 1987). These types of controls may be well exemplified by hormones and endocrine  
376 disruptors that may act on both positive and negative controls that regulate hormone action (Lagarde et  
377 al., 2015). Besides the biphasic dose-effect relationships, ionizing radiation may act by inducing a  
378 triphasic or a tetraphasic mode in the honeybee that can be also observed with pesticides in insects  
379 (Charpentier et al., 2014).

380 Abdominal GST did not appear to be modified by gamma rays during the experiment. Head AChE  
381 only showed significant correlations to dose rate at D3, but not after. In a similar way, citrus red mite,  
382 *Panonychus citri*, acutely exposed to gamma rays presented a decrease of AChE activity, but the  
383 values returned back to normal after 5 days of recovery (Zhang et al., 2014). This enzyme relates  
384 strongly to the action of organophosphorous insecticides (Badiou-Bénéteau et al., 2012b), but does not  
385 seem to be a relevant long-term biomarker for effects of gamma rays in bees. However, a decrease of  
386 AChE activity was shown in zebrafish larvae exposed to 0.8 mGy/d during 4 days, showing that  
387 gamma rays can have an impact on AChE, depending on the organism (Gagnaire et al., 2015).  
388 Intestinal CAT, PAL and GST varied with dose rate at D3 and D10, but not at D14; the values  
389 returned to normal by the end of the experiment, indicating a transitory effect of gamma rays.

390 Other biomarkers are more impacted by gamma rays. Abdominal CAT was not modified at D3 and  
391 D10, but at D14, a significant decreasing trend was shown with dose rates > 10 mGy/h. Both SODs  
392 globally presented an increasing trend at D3 and D14. CAT and SOD also showed significant  
393 relationships with dose rate. Hence, the gamma rays seemed to induce a general antioxidant response  
394 in honeybee. Ionizing radiation is known to induce oxidative stress. SOD and CAT activities were  
395 higher in mites (*P. citri*) submitted to an acute gamma-irradiation, but values returned to normal after a



396 recovery period (Zhang et al., 2014). Both enzymes also increased after acute irradiation exposure in  
397 *Chironomus ramosus* larvae (Datkhile et al., 2009). In fish, an increase of ROS basal levels also  
398 occurred in zebrafish larvae exposed to 0.8 mGy/d during 4 days, and modulation of the expression of  
399 myeloperoxidase gene was also observed (Gagnaire et al., 2015). After an acute irradiation, embryos  
400 of *K. marmoratus* also presented elevated basal ROS levels and an increase of several antioxidant  
401 enzymes including CAT, GST, GPx and SOD (Rhee et al., 2012). It would be interesting to measure  
402 the levels of ROS production in honeybees after gamma irradiation in order to confirm the results  
403 commonly observed on other species.

404 PO, an immunological biomarker, decreased significantly by D14 in irradiated bees and this decrease  
405 correlated significantly to dose rate. PO also decreased after acute gamma irradiation in fruit fly larvae  
406 (Mansour and Franz, 1996) and in *P. citri* (Zhang et al., 2014). Therefore, PO seems to be a relevant  
407 biomarker of effects of ionizing radiation in insects.

408 Concerning carboxylesterases, indicators of general metabolism, intestinal CaE1 and CaE2 showed a  
409 U-shape biphasic response at D3 and D14 but not at D10. Intestine CaE1 exhibited a triphasic (almost  
410 tetraphasic) response at D3, and CaE1 presented a similar triphasic profile at D14. Head CaE1, CaE2  
411 and CaE3 present a more complex response with a tetraphasic profile at D14. The response patterns  
412 observed were very time- and biomarker-dependent, without the expression of a unique pattern of  
413 response. Thus, it appears that carboxylesterases are modulated by gamma rays in the honeybee, but in  
414 a way difficult to understand from a biological point of view. Conversely, in *Apis cerana cerana*, a  
415 more obvious response can be observed, with an increase in the expression of carboxylesterase after  
416 UV radiation (Ma et al., 2018). Carboxylesterases seem to be particularly sensitive to pollutants or  
417 radiation in insects, molluscs and rodents with responses that are very specific because they are not  
418 associated to a modulation of tissue protein content (Auda et al., 1987; Badiou-Bénéteau et al., 2012a;  
419 Carvalho et al., 2013a; Fleming et al., 2016; Franco et al., 2016). Such a sensitivity to environmental  
420 stressors is not surprising because these enzymes are involved in numerous metabolic processes,  
421 hormone metabolism, reproduction and development, neural development or cell signaling (Hosokawa  
422 et al., 2007; Jackson et al., 2013; Khalil et al., 2006; Li et al., 2016; Vose et al., 2008). Hence, the

423 gamma rays could have effects on general metabolism, but other biomarkers could be more relevant to  
424 better understand their modes of action.

425 Gamma rays can induce DNA damages in vertebrate and invertebrate species (Adam-Guillermin et al.,  
426 2013). An interesting following of this work could be to study the DNA damages in honeybees  
427 exposed to ionizing radiation. The first step could be a comet assay that could be performed on  
428 hemocytes (Hayat et al., 2018).

429 In this study, we exposed honeybees to gamma rays and followed them for mortality for 14 days.  
430 Thus, long-term (>14 days) or delayed effects of exposure to gamma rays were not assessed. However,  
431 the longevity of a bee ranges from 20 to 50 days but the career of a forager ranges only from 8 to 11  
432 days before death (Neukirch, 1982; Wolf and Schmidhempel, 1989). Nevertheless, the honeybee  
433 queen has a lifespan of several years (generally 3-4 years) (Sammataro and Avitabile, 1998), which  
434 makes it a good bioindicator for the study of long-term effects of ionizing radiation.

435 In this study, we found that the physiology of the honeybee can be altered by a large range of ionizing  
436 radiation dose rates, without clear effects on mortality. Hence, subtle adverse mechanisms and effects  
437 can occur, even at low dose levels, thus revealing the sensitivity of the honeybee to ionizing radiation.  
438 Such discrete physiological modulations, in the absence of significant lethal effect, were also  
439 demonstrated with chemicals, like the insecticide fipronil, in the honeybee, which shows that stressors  
440 can impair physiological functions at low noise (Renzi et al., 2016). Thus, the honeybee can be used as  
441 a pertinent bioindicator not only to detect exposure to chemical pollutants, including pesticides  
442 (Badiou-Bénéteau et al., 2013b), but also to physical agents such as ionizing radiation or  
443 electromagnetic fields (Shepherd et al., 2018).

444 Globally, the enzymes of antioxidant and immune systems decreased with increasing dose rate.  
445 Reversible effects were shown on acetylcholinesterase. Concerning indicators of metabolism  
446 (carboxylesterases), variations occurred but no clear pattern was observed. However, the  
447 demonstration of the link between effects on biomarkers of several physiological functions and effects  
448 at the individual scale remains to be achieved. Indeed, a decrease of some immune and antioxidant

449 parameters can lead to an increase of susceptibility to diseases. In the same way, affections to general  
450 metabolism can lead to an acute vulnerability to nutritive demand. Moreover, some field studies  
451 showed that populations of bees declined in contaminated environments (Moller and Mousseau, 2009).  
452 An irradiation of several generations, even at low doses, might have more drastic effects than in a  
453 short period. Therefore, a next step of this work could be to place hives *in situ* in contaminated areas  
454 and to study the general behavior of bees (return to hive, number of entries/departure, time spent  
455 outside the hive, communication of a food source location), in order to understand the effects of  
456 ionizing radiation at the individual/population levels.

457

## 458 **Conclusion**

459 We investigated the sublethal effects of ionizing radiation on honeybees, *Apis mellifera*, using a  
460 battery of physiological biomarkers involved in metabolism, nervous system, immunity and  
461 antioxidant defenses. No excess of mortality was observed, but several physiological markers involved  
462 in antioxidant (CAT, SOD) and immune (PO) systems were significantly correlated to the external  
463 dose rate. These biomarkers may be the targets of early effects of exposure to gamma rays in bees.  
464 However, they are not specific of exposure to radiation, as they can also be modulated by other  
465 pollutants including pesticides.

466 Our study helped to improve the knowledge on the mechanisms of action of gamma rays in insects,  
467 using the honeybee as a model species. This kind of approach is necessary in order to accumulate data  
468 that could be used in the assessment of the environmental risk posed by ionizing radiation on  
469 ecosystems. A perspective of this work could be using bees and hives as biomonitoring tools of  
470 contaminated sites (Chernobyl, Fukushima) or around nuclear power plants in order to assess their  
471 impact on ecosystems.

## 472 **Acknowledgments**

473 The authors would like to thank ECCOREV research federation for the funding of this project.

474 **Figure captions**

475 **Fig. 1. Effect of the dose rate of irradiation on the physiological markers at D3**

476 Here are presented only markers whose modulation was complex. The dose-effect relationships were  
477 fitted with polynomial functions of order 2 for A, order 3 for B, C and D, and order 4 for E and F. H, I  
478 and A represent the organ considered (head, intestine and abdomen, respectively). CaE1 and CaE2:  
479 carboxylesterases 1 and 2; AChE: acetylcholinesterase; ALP: alkaline phosphatase; SOD: superoxide  
480 dismutase; CAT: catalase. Each data represents the mean value of three replicates performed on pools  
481 of 5 organs (seven pools for the controls and for dose rates of 210 and 588 mGy/d, and four pools for  
482 other conditions). Grey areas represent the 95% confidence intervals. All data are expressed in  
483 Absorbance Units/min/mg of tissue.

484

485 **Fig. 2. Effect of the dose rate of irradiation on the physiological markers at D10**

486 Here are presented only markers whose modulation was complex. The dose-effect relationships were  
487 fitted with polynomial functions of order 2 for A and order 3 for B. H represents the organ considered  
488 (head). CaE3: carboxylesterase 3; GPx: glutathione peroxidase. Each data represents the mean value of  
489 three replicates performed on pools of 5 organs (seven pools for the controls and for dose rates of 210  
490 and 588 mGy/d, and four pools for other conditions). Grey areas represent the 95% confidence  
491 intervals. All data are expressed in Absorbance Units/min/mg of tissue.

492

493 **Fig. 3. Effect of the dose rate of irradiation on the physiological markers at D14**

494 Here are presented only markers whose modulation was complex. The dose-effect relationships were  
495 fitted with polynomial functions of order 3. H, I and A represent the organ considered (head, intestine  
496 and abdomen). CaE1 and CaE2: carboxylesterases 1 and 2; SOD: superoxide dismutase; CAT:  
497 catalase. Each data represents the mean value of three replicates performed on pools of 5 organs

498 (seven pools for the controls and for dose rates of 210 and 588 mGy/d, and four pools for other  
499 conditions). Grey areas represent the 95% confidence intervals. All data are expressed in Absorbance  
500 Units/min/mg of tissue.

501

502 **Fig. 4. Effect of the dose rate of irradiation on the physiological markers at D14**

503 Here are presented only markers whose modulation was complex. The dose-effect relationships were  
504 fitted with polynomial functions of order 4. H and A represent the organ considered (head and  
505 abdomen). CaE1, Ca E2 and CaE3: carboxylesterases 1, 2 and 3; CAT: catalase; PO: phenoloxidase.  
506 Each data represents the mean value of three replicates performed on pools of 5 organs (seven pools  
507 for the controls and for dose rates of 210 and 588 mGy/d, and four pools for other conditions). Grey  
508 areas represent the 95% confidence intervals. All data are expressed in Absorbance Units/min/mg of  
509 tissue.

510

511 **Fig. 5. PCA analysis performed on all physiological markers**

512 (A), D3; (B), D10. (C), D14. H, I and A represent the organ considered (head, intestine and abdomen).  
513 Dose rate was included as a quantitative supplementary variable. For biomarker denomination, see  
514 legends of Figures 1-4.

515

516 **Fig. 6. Cluster analysis performed on all physiological biomarkers**

517 (A), D3; (B), D10. (C), D14. H, I and A represent the organ considered (head, intestine and abdomen).  
518 For biomarker denomination, see legends of Figures 1-4.

519

520

521 **Table 1. Significance levels (*p*-value) obtained for the biomarkers that fitted a linear model.**

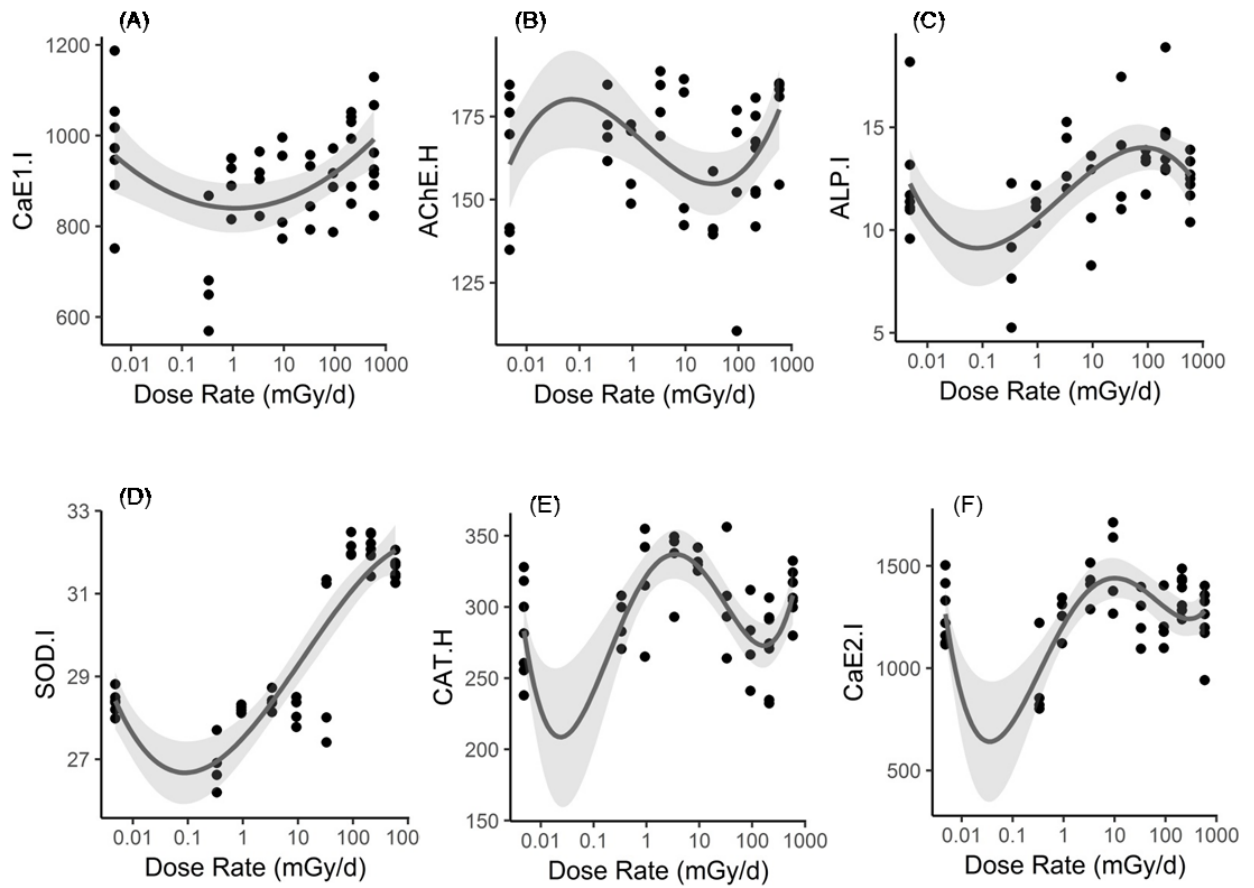
	Head					Intestine			Abdomen
	CaE1.H	CaE2.H	CaE3.H	CAT.H	GPx.H	CaE3.I	CAT.I	GST.I	SOD.A
<b>D3</b>	0.036	NS	0.0017	NR	NS	NS	0.01	<b>0.007</b>	0.026
<b>D10</b>	<b>9x10<sup>-4</sup></b>	<b>9.7x10<sup>-8</sup></b>	NR	<b>0.029</b>	NR	<b>0.0017</b>	0.006	4x10 <sup>-4</sup>	NS
<b>D14</b>	NR	NR	NR	NR	<b>0.04</b>	<b>0.0019</b>	NS	NS	NR

522

523 The significance levels were indicated at different times (D3, D10 and D14) for the markers of  
 524 interest. Bold values indicated negative relationships between the biomarker activity and the dose rate;  
 525 non-bold values indicated positive relationships between the biomarker activity and the dose rate. NR,  
 526 not relevant (non-linear fitting); NS: linear-fitting, but not statistically significant.

527

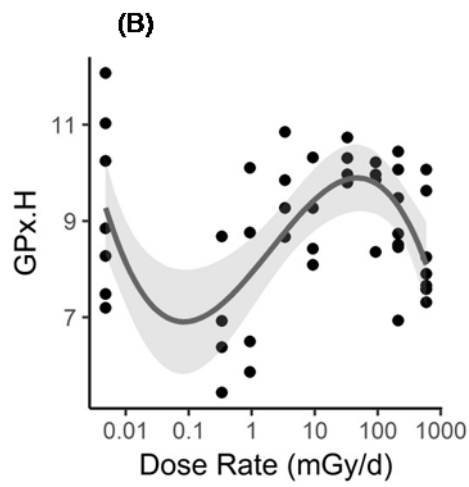
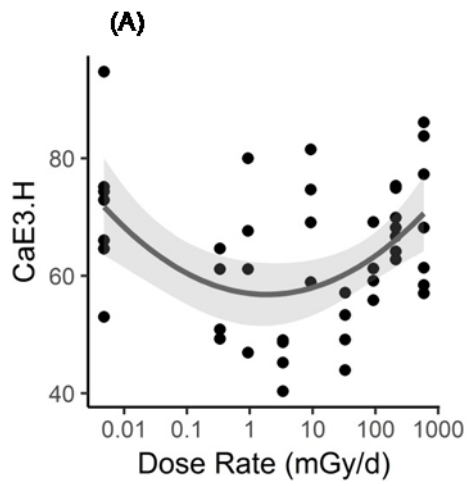
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530 **Fig. 1**

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535 **Fig. 2**

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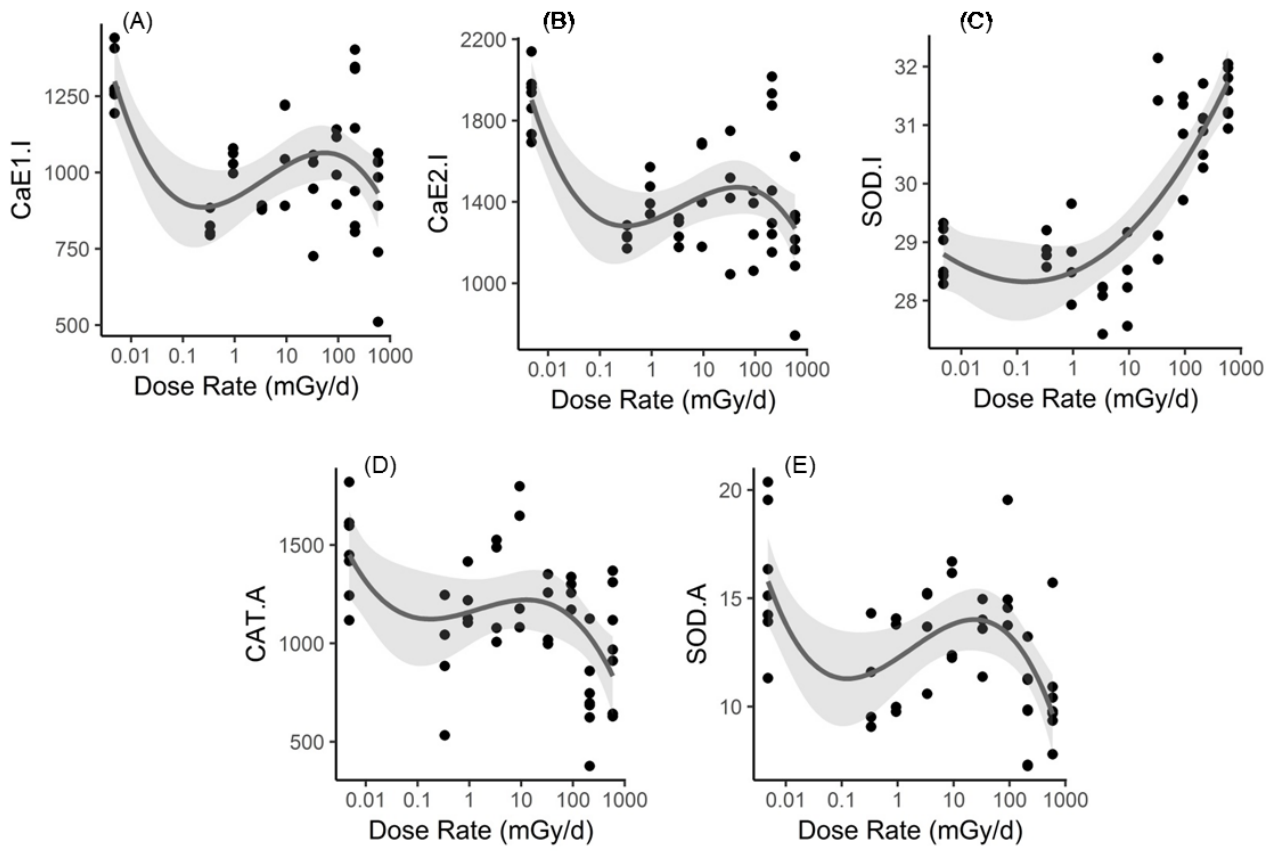
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546 **Fig. 3**

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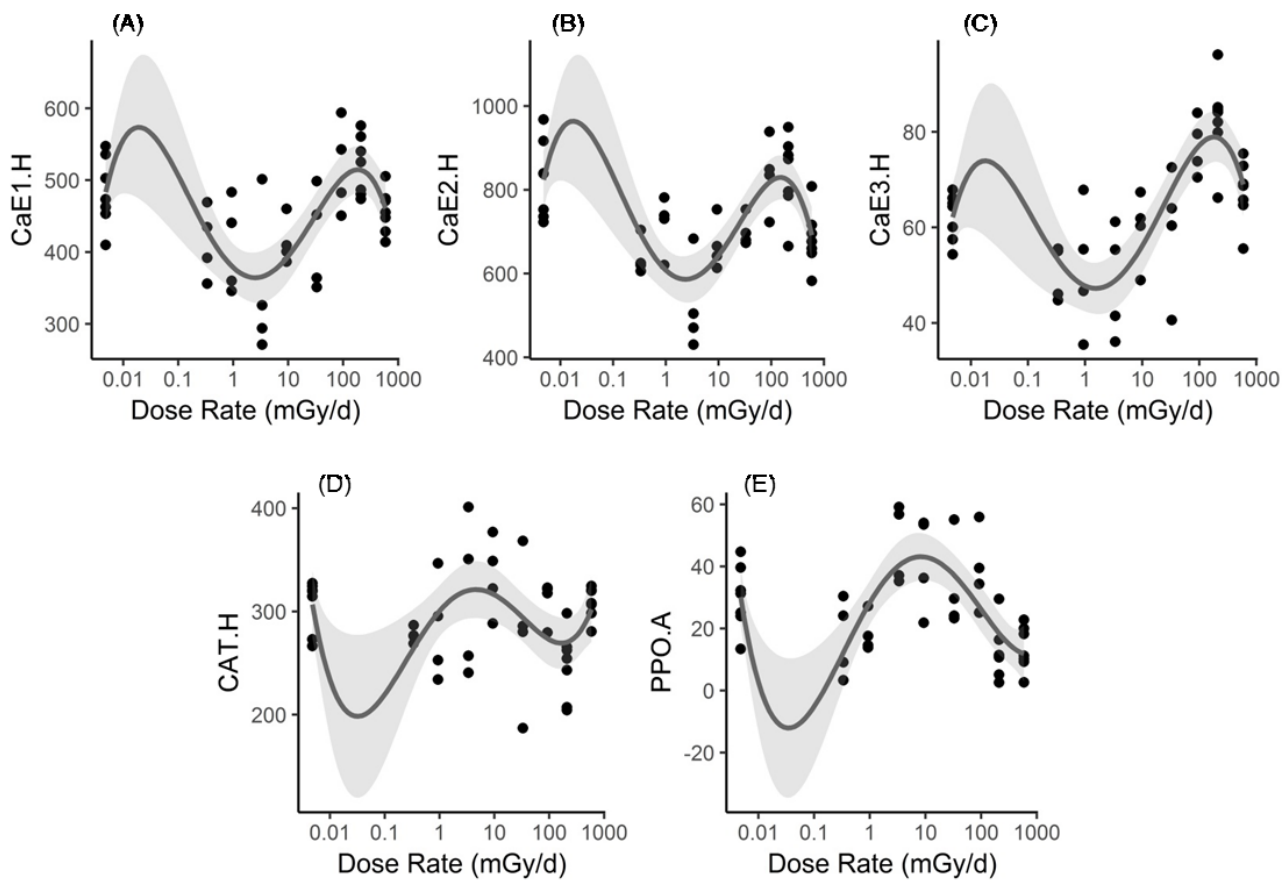
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557 **Fig. 4**

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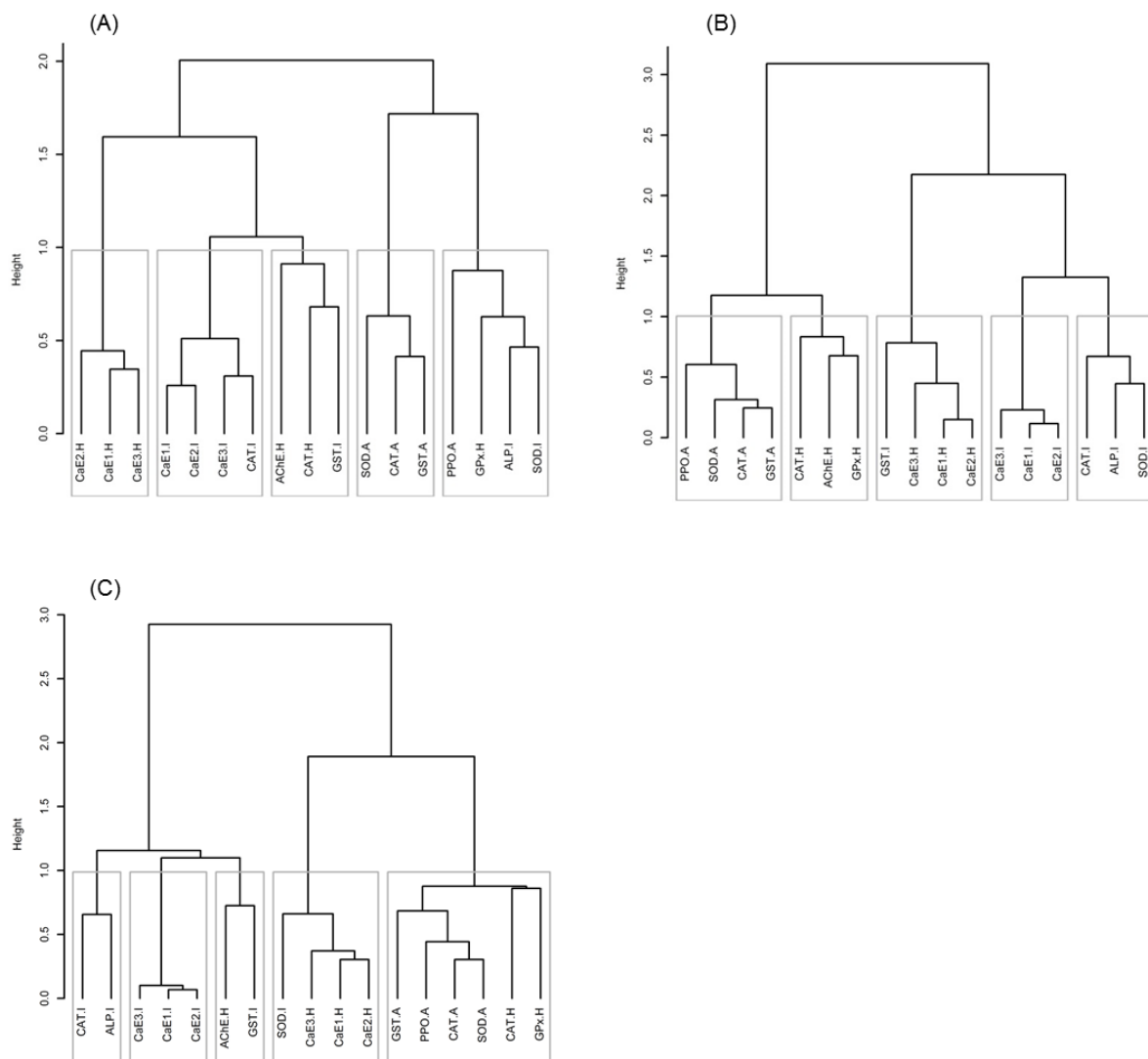
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563





586 **Fig. 6**

589 **SUPPLEMENTARY MATERIALS**

590

591 **Figure captions**

592 **Fig. S1. Plots of the biomarkers at D3 that presented a significant linear relationship with the**  
593 **dose rate**

594 H, I and A represent the organ considered (head, intestine and abdomen). For biomarker  
595 denomination, see legends of Figures 1 -4. Each data represents the mean value of three replicates  
596 performed on pools of 5 organs (seven pools for the controls and for dose rates of 210 and 588 mGy/d,  
597 and four pools for other conditions). All data are expressed in mAU/min/mg of tissue.

598

599 **Fig. S2. Plots of the biomarkers at D10 that presented a significant linear relationship with the**  
600 **dose rate**

601 H and I represent the organ considered (head and intestine). For biomarker denomination, see legends  
602 of Figures 1-4. Each data represents the mean value of three replicates performed on pools of 5 organs  
603 (seven pools for the controls and for dose rates of 210 and 588 mGy/d, and four pools for other  
604 conditions). Grey areas represent the 95% confidence intervals. All data are expressed in Absorbance  
605 Units/min/mg of tissue.

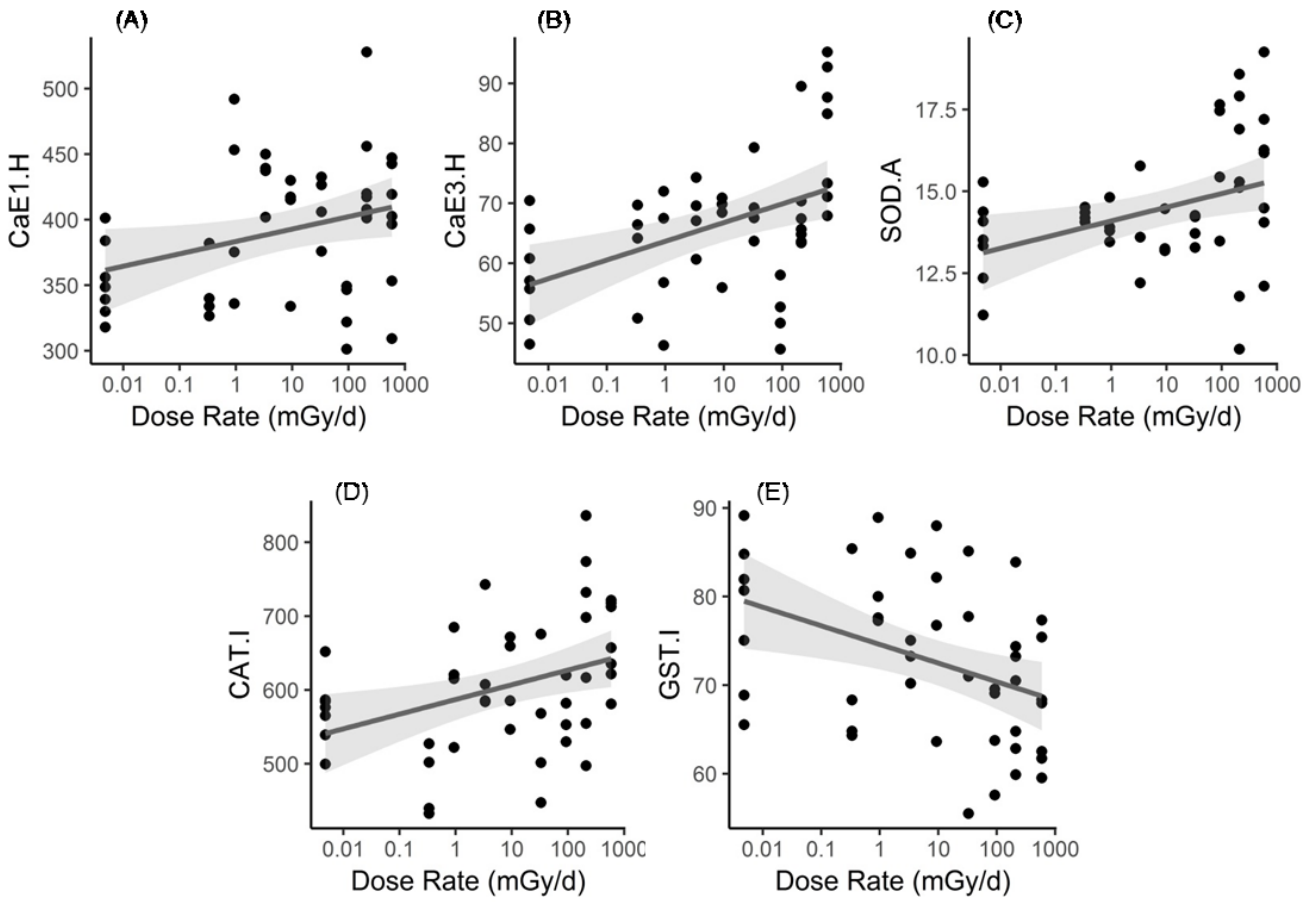
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607 **Fig. S3. Plots of the biomarkers at D14 that presented a significant linear relationship with the**  
608 **dose rate**

609 H and I represent the organ considered (head and intestine). For biomarker denomination, see legends  
610 from Figures 1 to 4. Each data represents the mean value of three replicates performed on pools of 5  
611 organs (seven pools for the controls and for dose rates of 210 and 588 mGy/d, and four pools for other

612 conditions). Grey areas represent the 95% confidence intervals. All data are expressed in Absorbance  
613 Units/min/mg of tissue.

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618 **Figure S1**

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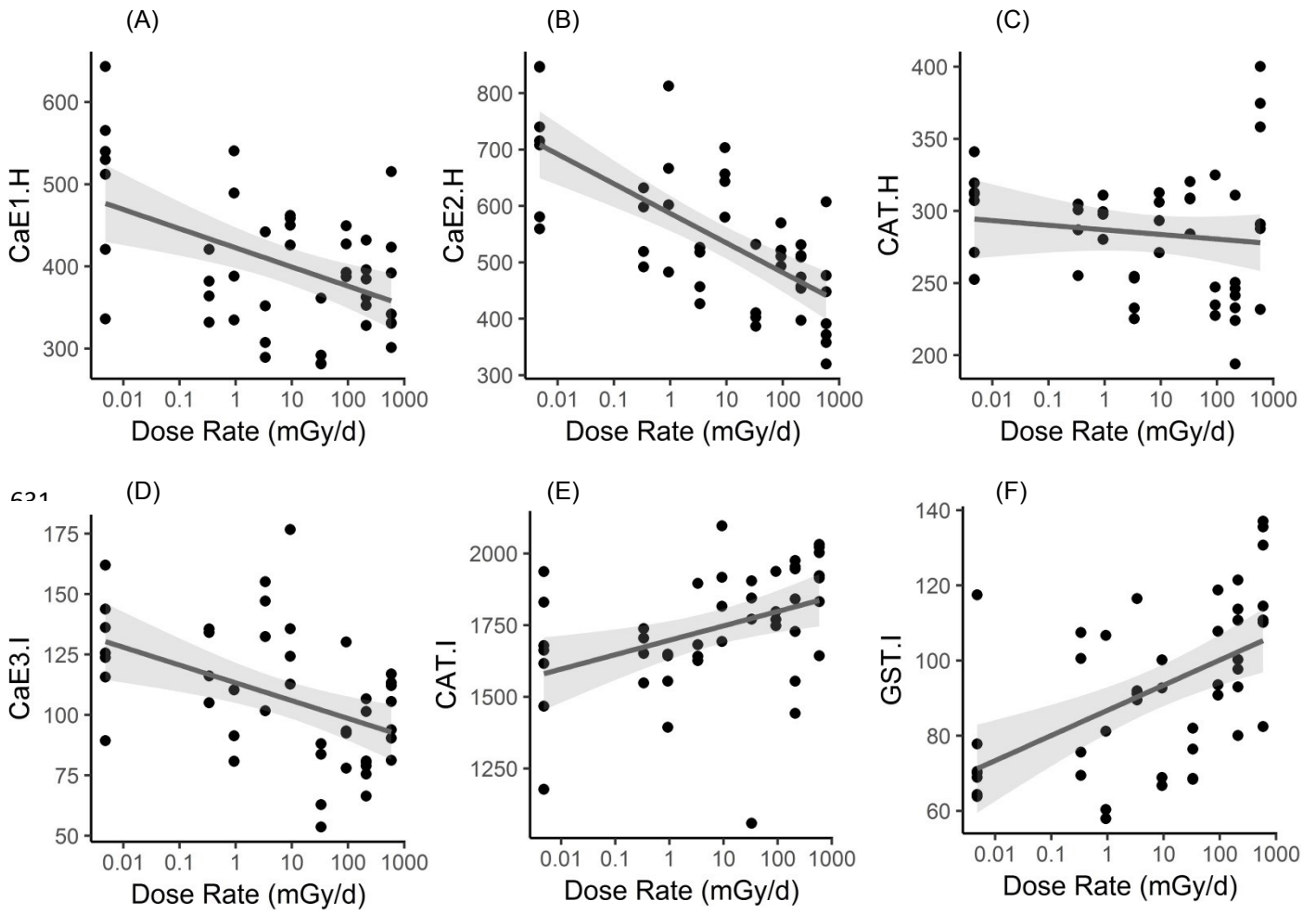
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636 Erreur ! Source du renvoi introuvable.

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639 **Figure S2**

640

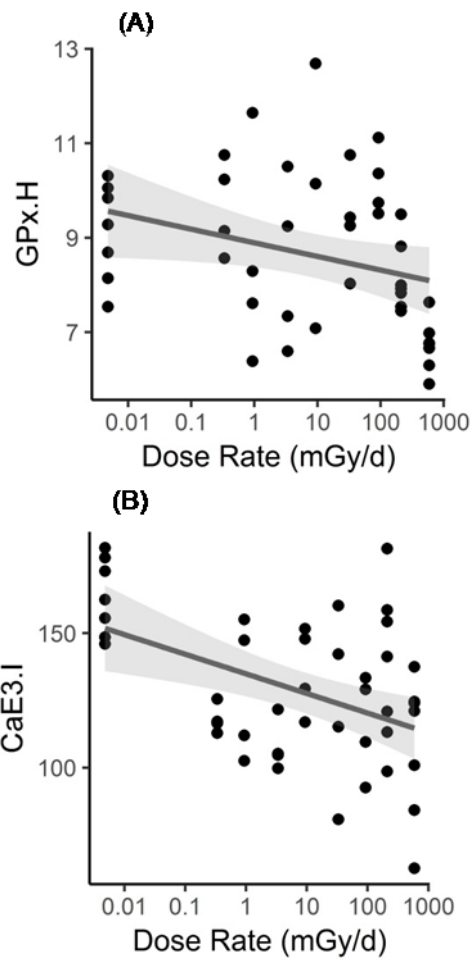
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647 **Figure S3**

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