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

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Abstract

Terrestrial ecosystems are exposed to various kinds of pollutants, including radionuclides. The honeybee, *Apis mellifera*, is commonly used in ecotoxicology as a model species for evaluating the effects of pollutants. In the present study, honeybees were irradiated right after birth for 14 days with gamma rays at dose rates ranging between $4.38x10^{-3}$ and 588 mGy/d. Biological tissues (head, intestine and abdomen) were sampled at D3, D10 and D14. Ten different physiological markers involved in nervous (acetylcholinesterase (AChE)), antioxidative (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST)), immune system (phenoloxidase (PO)) and metabolism (carboxylesterases (CaEs) and alkaline phosphatase (ALP)) were measured. Univariate analyses were conducted to determine whether each individual biomarker response was positively or negatively correlated with the dose rate. Then, multivariate analyses were applied to investigate the relationships between all the biomarker responses. Although no mortality occurred during the experiment, several biomarkers varied significantly in relation to the dose rate. Globally, the biomarkers of antioxidant and immune systems decreased as the dose rate increased. Reversible effects on the indicator of the neural system were found. Concerning indicators of metabolism (carboxylesterases), variations occurred but no clear pattern was found. Taken altogether, these results help better understand the effects of ionizing radiation on bees by identifying relevant physiological markers of effects. These results could improve the assessment of the environmental risk due to ionizing radiation in terrestrial ecosystems.

Keywords

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

Introduction

Protecting the environment in the context of global change and sustainable management of resources and ecosystems is a major concern worldwide. Environmental pollution is a major problem for human, animal and plant populations (Colosio et al., 2005). Among the different pollutants, radioactive elements, such as uranium, can occur naturally. However, artificial radionuclides can also be released by human activities through normal functioning conditions of nuclear fuel cycle installations, controlled wastes from industrial and nuclear medicine activities, nuclear waste storage sites, deposits from nuclear tests or nuclear accidents, such as those that occurred at Chernobyl and Fukushima. These releases lead to a worldwide background of absorbed dose rate ranging from $5x10^{-4}$ to $4x10^{-3}$ mGy/d in the environment, depending on the geographic zone (UNSCEAR, 1996). This radioactivity can increase in accidental contexts, with absorbed dose rates of 24 mGy/d in the case of the Fukushima accident (Adam-Guillermin et al., 2016).

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

Ecotoxicological properties of ionizing radiation have not been extensively studied for nonhuman species, particularly for terrestrial invertebrates such as bees. Information regarding exposure to ionizing radiation in bees is limited to bioaccumulation data (Fresquez et al., 1997; Haarmann, 1997, 1998a, b; Hakonson and Bostick, 1976). Further, information on mechanisms of toxicity, early and/or sublethal effects of exposure to ionizing radiation are scarce, despite the importance of bees for ecosystem sustainability. However, some field studies have revealed that populations of bumble-bees, 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 $1x10^3$
131 mGy/d and reproductive success can possibly be reduced for dose rates of $1x10^2$-$1x10^3$ mGy/d (ICRP,
132 2008). The Dose Reference Consideration Levels (DCRL) for bees, for which deleterious effects are
133 expected, are between 1x10 and $1x10^2$ 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}Cs) at dose rates
136 ranging from $4.38x10^{-3}$ (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

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

Material and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). The chemicals included: antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monobasic and dibasic sodium phosphate, sodium chloride (NaCl), triton X-100, acetylthiocholine iodide, *5*,*5'*-dithio-bis(*2*-nitrobenzoic acid) (DTNB), α- and β-naphthyl acetate (α-NA or β-NA), *p*-nitrophenyl acetate (*p*-NPA), *1*,*5*-bis (*4*-allyldimethylammonium-phenyl)pentan-*3*-one-dibromide (BW284C51), fast garnet GBC, sodium dodecyl sulfate (SDS), hydrogen peroxide (H_2O_2), monobasic potassium phosphate, ethylenediaminetetraacetic acid (EDTA), *1*-chloro-*2*,*4*-dinitrobenzene (CDNB), reduced L-glutathione (GSH), oxidized glutathione (GSSG), acetonitrile, acetone, NADPH, tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), *p*-nitrophenyl phosphate (*p*-NPP), *tert*-butyl hydroperoxide (TBHP), *3*,*4*-Dihydroxy-L-phenylalanine (L-DOPA) and bovine serum albumin.

Honeybees

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

ad libitum with candy paste and water. The insects were kept at 32 ± 2 °C and 60 ± 10% relative humidity.

Exposure to gamma rays

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

Every day, dead honeybees were removed from the cages and daily food consumption was measured and expressed as percentages of food consumption during the first day in controls. Living honeybees were randomly selected and removed at D0, D3, D10 and D14, and tissues were sampled and immediately frozen at -80°C until biomarker analysis.

Tissue extracts

To prevent any animal suffering, all tissues were removed from bees previously anesthetized and then decapitated. Honeybee heads were obtained by cutting from the body with a scalpel. Then, midguts were obtained by pulling the stingers from the honeybees. Abdomens correspond to abdomens devoid of intestinal tract. Tissues samples (pools of tissues from 5 bees) were placed in a 2-mL microfuge tubes. In order to provide enough material for analyses and to limit inter-individual variability, seven pools were made for controls and bees exposed at 210 and 588 mGy/d; four pools were made for the other conditions. The extraction buffer was added to make a 10% (w/v) tissue extract. The extraction buffer consisted of 1% (w/v) Triton X-100, 10 mM sodium chloride and 40 mM sodium phosphate at pH 7.4, and contained protease inhibitors (2 mg/mL antipain, leupeptin, and pepstatin A, 25 units/mL aprotinin, and 0.1 mg/mL soybean trypsin inhibitor) (Belzunces et al., 1988). The tissues were grinded 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 α-naphtyl acetate (α-NA), β-naphtyl
201 acetate (β–NA) and *p*-nitrophenyl acetate (p-NA), respectively (Gomori, 1953). The crude tissue
202 extract was incubated in a medium containing $1x10^{-4}$ 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 (α–NA, β–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 α-NA (CaE1) and 515 nm for β-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 $MgCl_2$, 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_2O_2 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 (log10) because dose rate was highly spread (ratio max/min = 1.2×10^6) 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.

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

Results

Mortality and feeding

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

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

Relationship between physiological marker levels and dose rate

At D3, the activity of several physiological markers showed a significant positive linear relationship with the dose rate: that was the case for head CaE1 and CaE3, intestinal CAT and abdominal SOD (Table 1, Figure S1). However, intestinal GST presented a significant negative linear relationship with the dose rate (Table 1, Figure S1). Head CaE2 and GPx, intestinal CaE3 and abdominal CAT, GST and PO did not have linear significant relationships with the dose rate (data not shown). Other physiological markers presented complex and non-monotonic significant relationships with the dose rate. A two-order polynomial relationship was found for intestinal CaE1, with a decrease of activity 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 that 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 that 1 mGy/d an 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 that 0.1 mGy/d (Fig. 3C). Finally, several
290 physiological markers were related to the dose rate with a four-order polynomial relationship. Head

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

Multivariate analyses

The relationships between all of the biochemical biomarkers were analysed with PCA performed on the whole set of data for each sampling time (Fig. 5). For D3, the two first axes explained 40% of inertia. The first axis was explained by the three CaE and CAT in the intestine. To a lesser degree, the first axis was also explained by all head CaE. The second axis was explained by high values of intestinal and abdominal GST and abdominal and head CAT. Negative values of second axis were related to high values of intestinal SOD and ALP. Dose rate was poorly related to both axes (Fig. 5A). For D10, the two first axes explained 41% of inertia. The contribution of intestinal and head CaE on the first axis was similar to D3. Intestinal CAT was less related to the first axis, but intestinal SOD was more strongly related to the first axis. The second axis was very well explained by abdominal SOD, CAT and GST, which were very correlated each other, and also in a lower manner by abdominal PO and head GPx. As for D3, dose rate was poorly related to both axes (Fig. 5B). For D14, the two first axes explained 43% of inertia. The contribution of biomarkers to axes was different compared to D3 and D10. The first axis was still explained mainly by abdominal biomarkers (SOD, CAT and PO), while the second axis explained primarily by head biomarkers (CaE1, CaE2, CaE3). However, at D14, intestinal biomarkers were related to both axes. Moreover, abdominal PO and intestinal SOD were negatively correlated. Dose rate was associated with low values of the abdominal biomarkers contributing to first axis (Fig. 5B). For all sampling times, the three CaE presented high correlations 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_{50} 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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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

Acknowledgments

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

Figure captions

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

Here are presented only markers whose modulation was complex. The dose-effect relationships were 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 and A represent the organ considered (head, intestine and abdomen, respectively). CaE1 and CaE2: carboxylesterases 1 and 2; AChE: acetylcholinesterase; ALP: alkaline phosphatase; SOD: superoxide dismutase; CAT: catalase. Each data represents the mean value of three replicates performed on pools of 5 organs (seven pools for the controls and for dose rates of 210 and 588 mGy/d, and four pools for other conditions). Grey areas represent the 95% confidence intervals. All data are expressed in Absorbance Units/min/mg of tissue.

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

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

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

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

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

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

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

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

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

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

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

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**
D3	0.036	NS	0.0017	NR	NS	NS	0.01	**0.007**	0.026
D10	**$9x10^{-4}$**	**$9.7x10^{-8}$**	NR	**0.029**	NR	**0.0017**	0.006	$4x10^{-4}$	NS
D14	NR	NR	NR	NR	**0.04**	**0.0019**	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.

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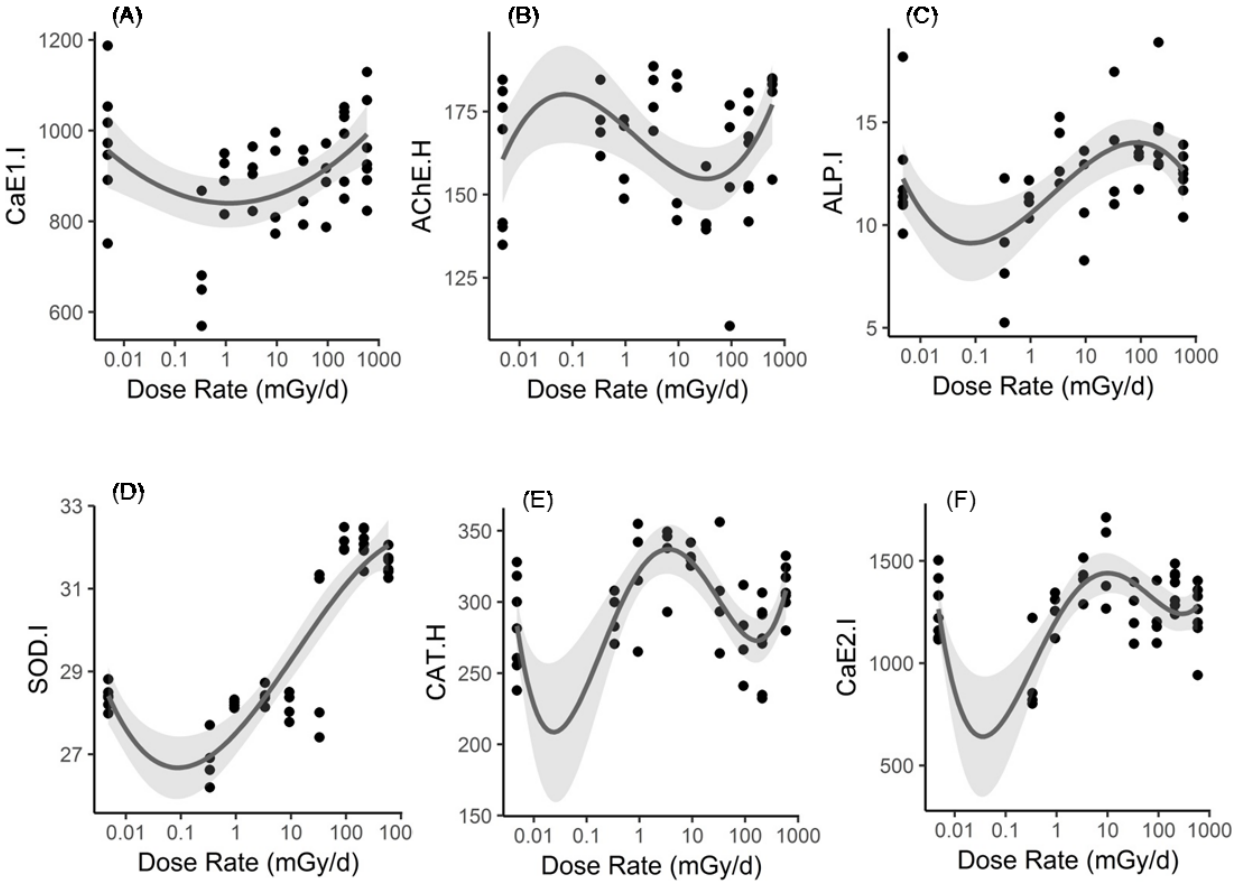

529

530 **Fig. 1**

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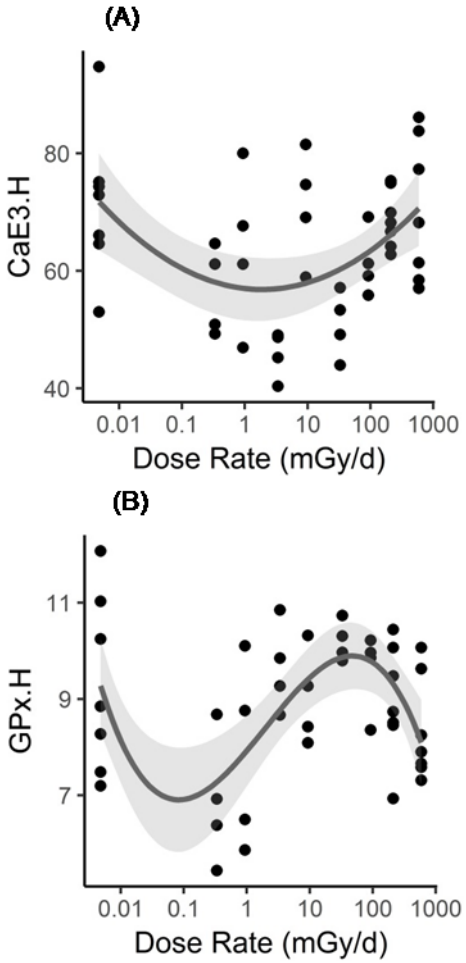

(A)
CaE3.H
80
60
40
0.01
0.1
1
10
100
1000
Dose Rate (mGy/d)
(B)
GPx.H
11
9
7
0.01
0.1
1
10
100
1000
Dose Rate (mGy/d)

Fig. 2

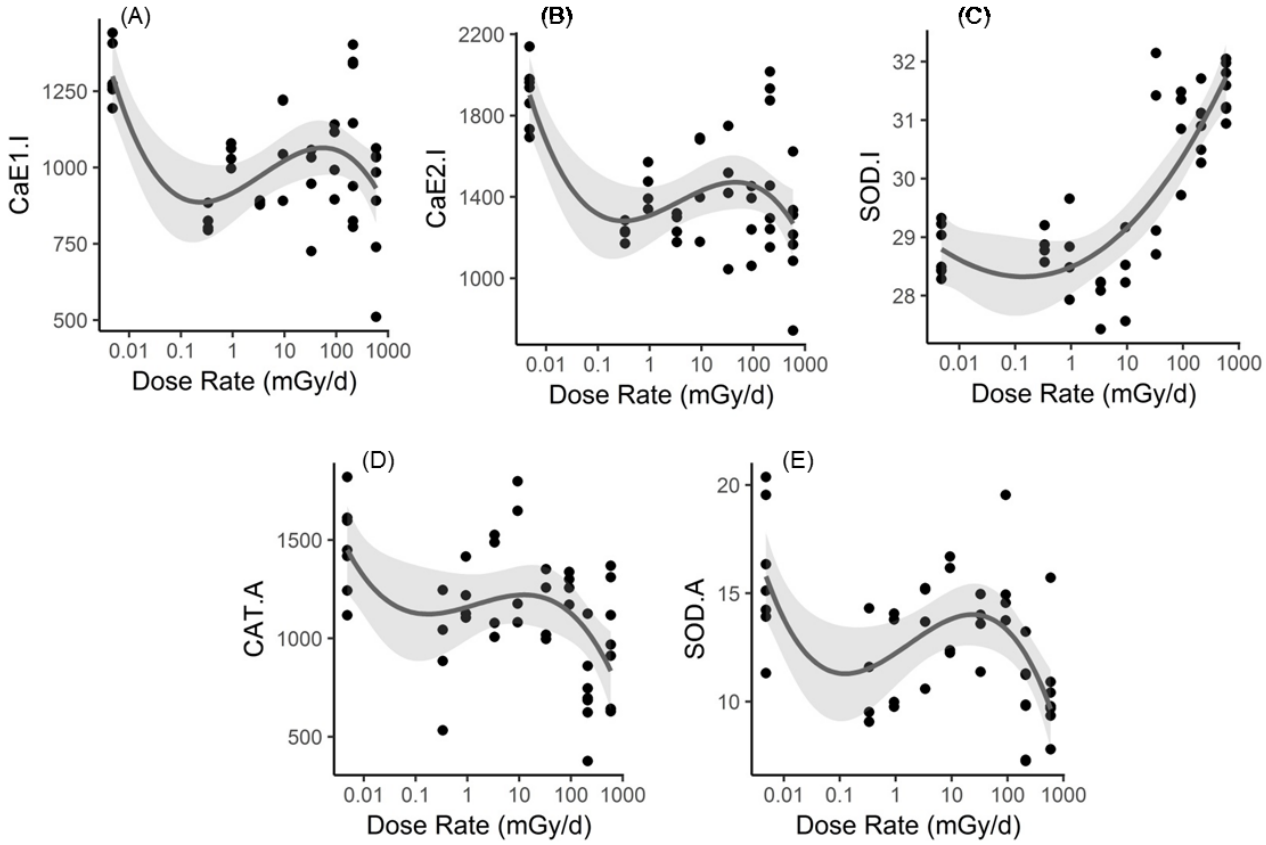
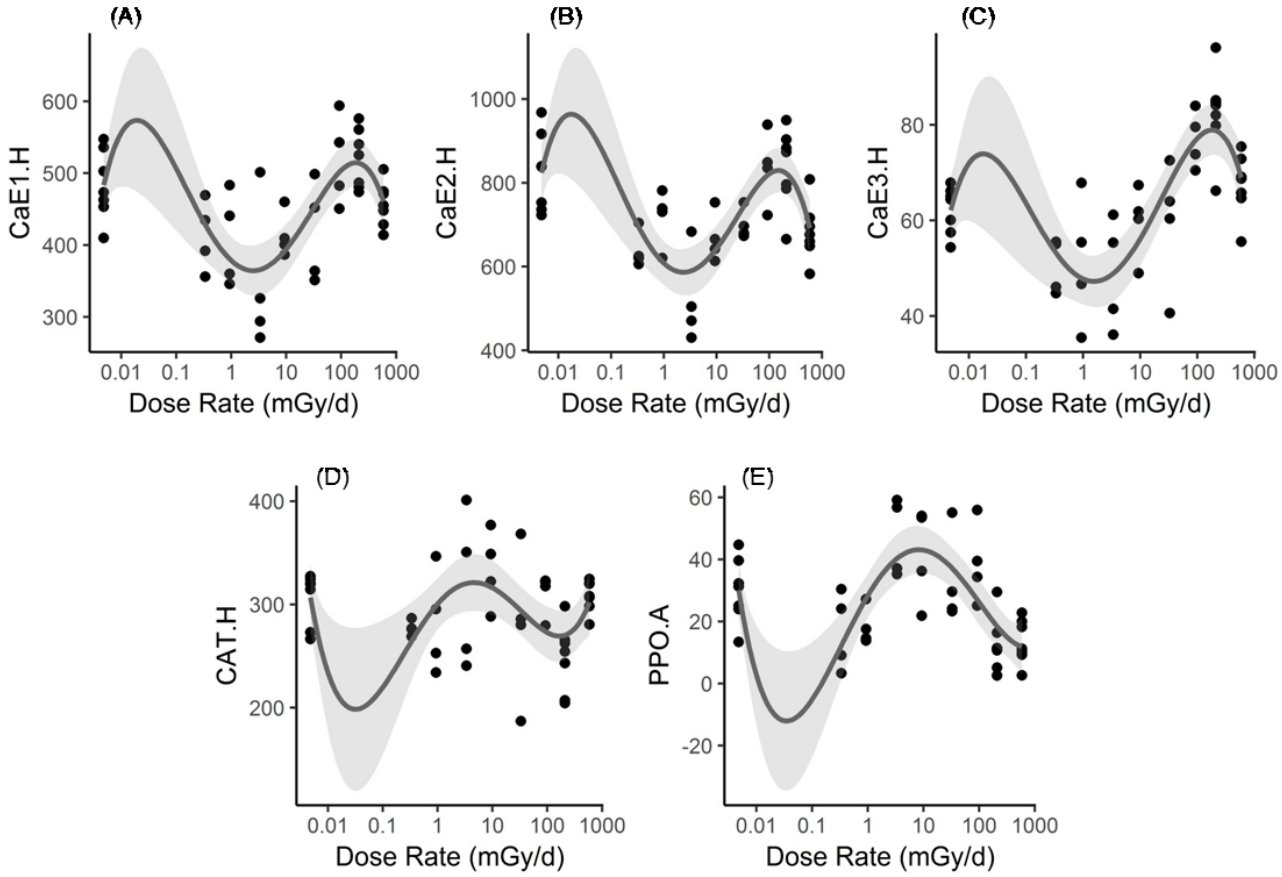

(A)
CaE1.I
1250
1000
750
500
0.01 0.1 1 10 100 1000
Dose Rate (mGy/d)
(B)
CaE2.I
2200
1800
1400
1000
0.01 0.1 1 10 100 1000
Dose Rate (mGy/d)
(C)
SOD.I
32
31
30
29
28
0.01 0.1 1 10 100 1000
Dose Rate (mGy/d)
(D)
CAT.A
1500
1000
500
0.01 0.1 1 10 100 1000
Dose Rate (mGy/d)
(E)
SOD.A
20
15
10
0.01 0.1 1 10 100 1000
Dose Rate (mGy/d)

Fig. 3



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557 **Fig. 4**

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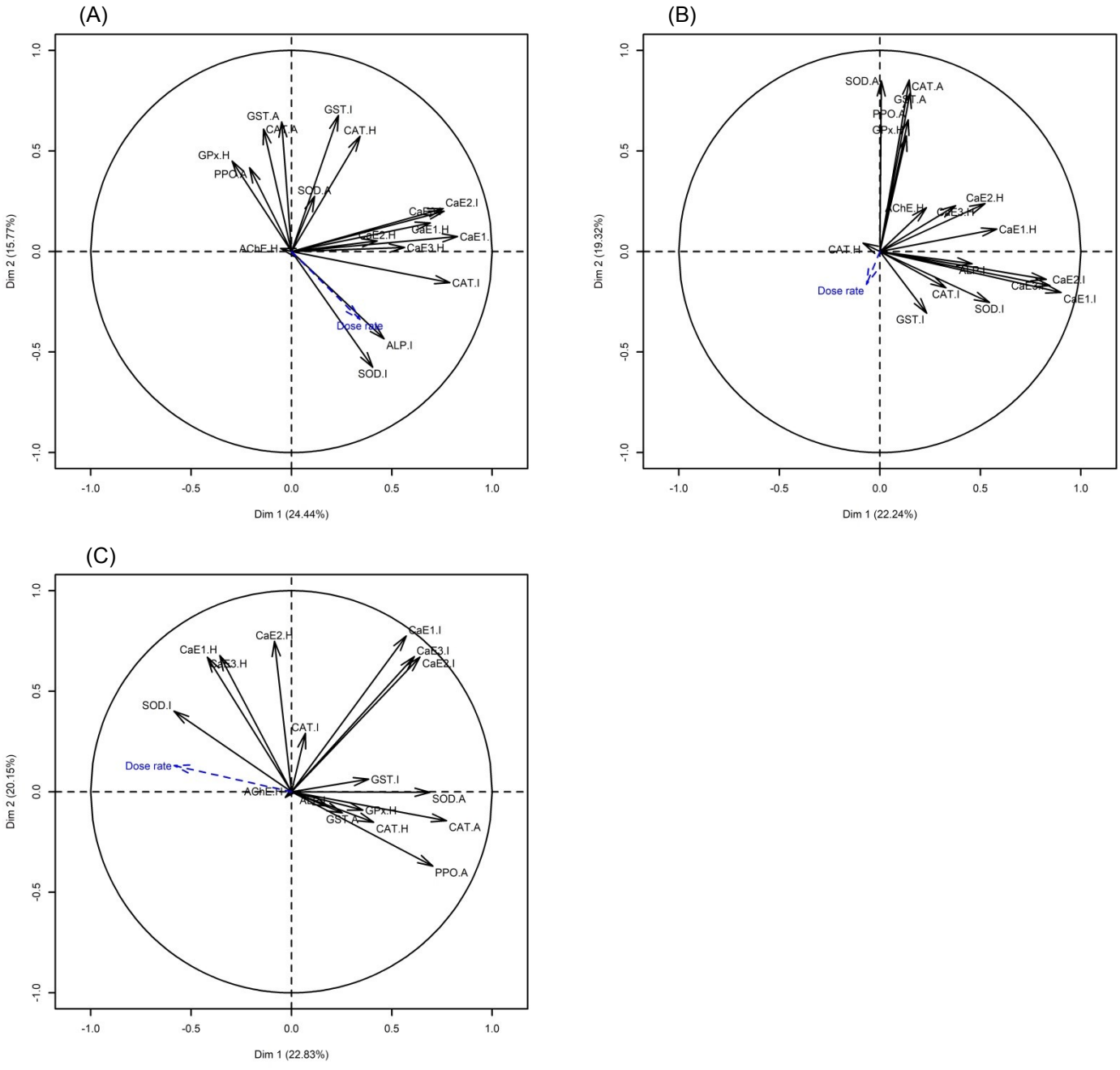

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580 **Fig. 5**

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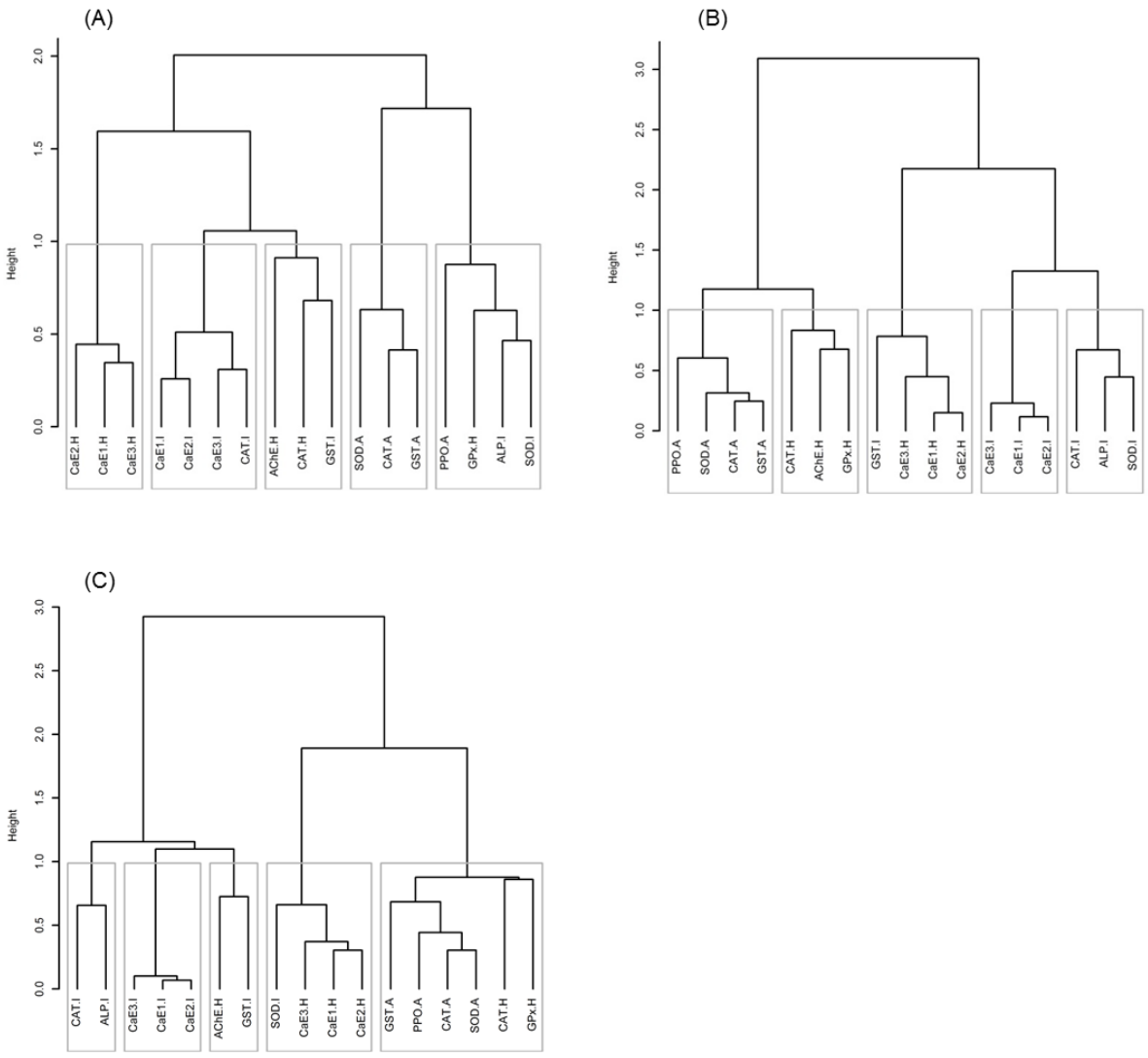

585

586 **Fig. 6**

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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.

606

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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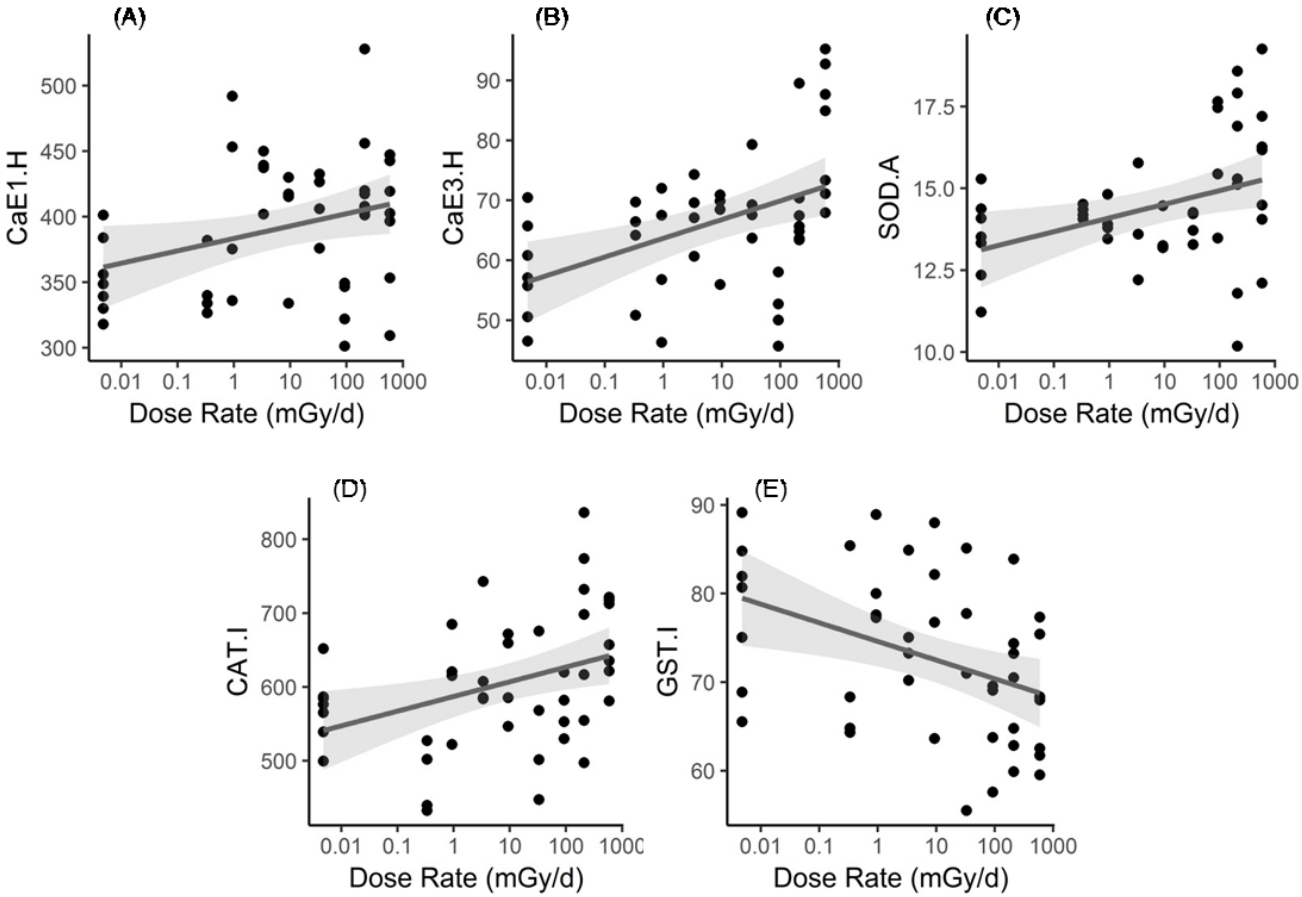

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618 **Figure S1**

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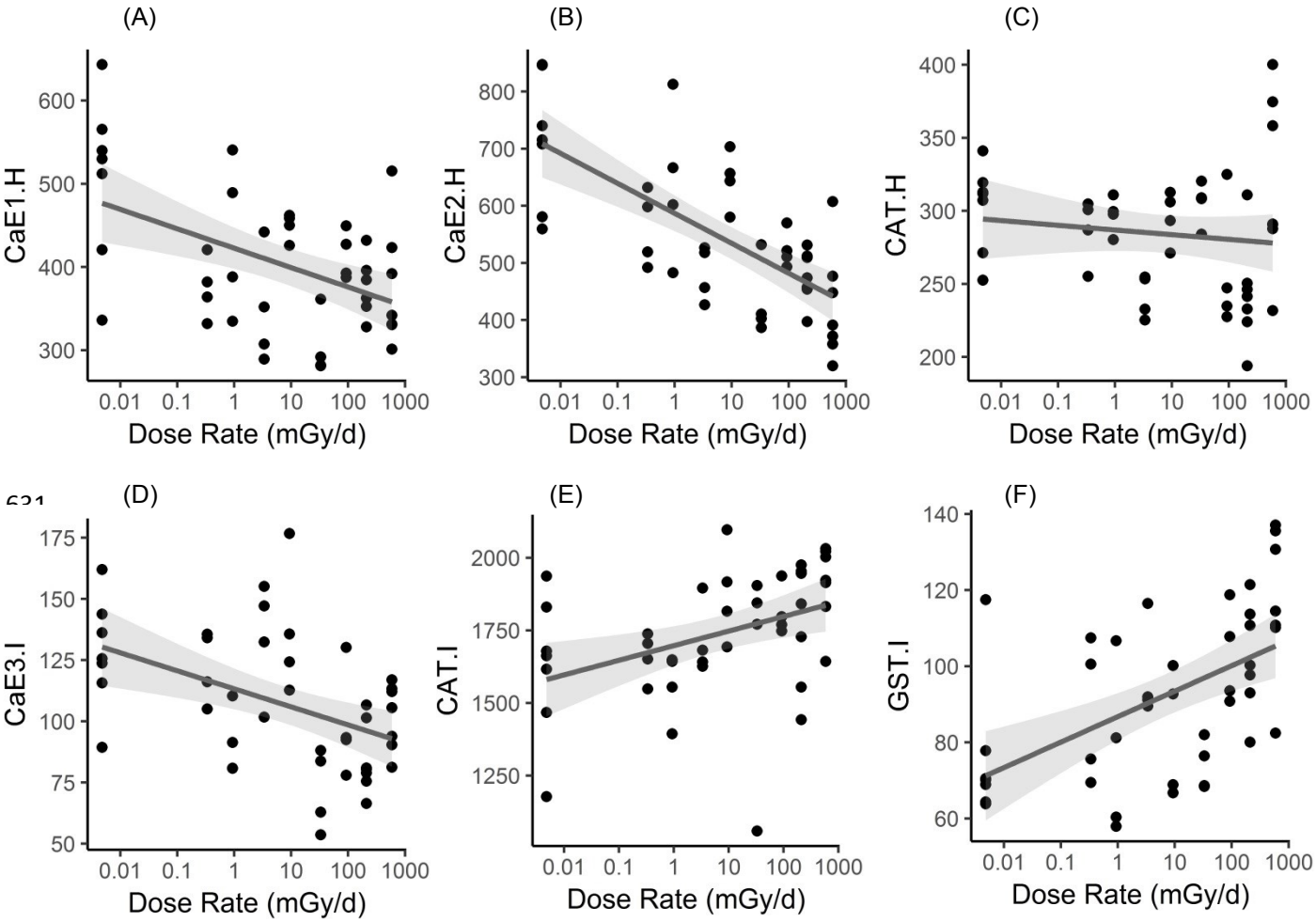

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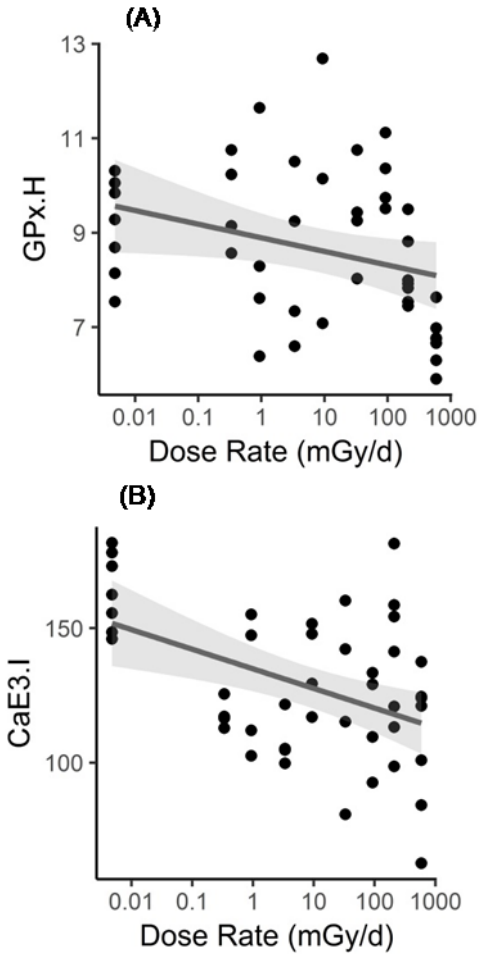
Figure S2



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647 **Figure S3**

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