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Effects of in vivo exposure to tritium: a multi-biomarker approach using the fathead minnow, *Pimephales promelas*

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1 **Effects of *in vivo* exposure to tritium: a multi-biomarker approach using the fathead minnow,**
2 ***Pimephales promelas***

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24 **Highlights**

- 25 - Following a previously published field investigation, this study aims to characterize tritium
- 26 effects on fish health under controlled conditions in a laboratory setting.
- 27 - Fathead minnows were exposed to tritium activity concentrations up to 180,000 Bq/L.
- 28 - At the highest levels of exposure, tritium increased DNA damage and modulated the immune
- 29 responses.
- 30 - Other markers were affected, including the neural system, oxidative stress and fatty acid
- 31 composition.
- 32 - No effects are reported on the measured health indices and anti-oxidant activities.

33

34 **Abstract**

35 Tritium (^3H) is a radioactive isotope of hydrogen. In the environment, the most common form of
36 tritium is tritiated water (HTO). However, tritium can also be incorporated into organic molecules,
37 forming organically bound tritium (OBT). The present study characterized the effects of tritium on the
38 health of the fathead minnow, *Pimephales promelas*. Fish were exposed to a gradient of HTO (activity
39 concentrations of 12,000, 25,000 and 180,000 Bq/L) and OBT using food spiked with tritiated amino
40 acids (OBT only, with an activity concentration of 27,000 Bq/L). A combined exposure condition
41 where fish were placed in 25,000 Bq/L water and received OBT through feed was also studied. Fish
42 were exposed for 60 days, followed by a 60 day depuration period. A battery of health biomarkers
43 were measured in fish tissues at seven time points throughout the 120 days required to complete the
44 exposure and depuration phases. HTO and OBT were also measured in fish tissues at the same time
45 points. Results showed effects of increasing tritium activity concentrations in water after 60 days of
46 exposure. The internal dose rates of tritium, estimated from the tissue free-water tritium (TFWT) and
47 OBT activity concentrations, reached a maximum of 0.65 $\mu\text{Gy/h}$, which is relatively low considering
48 background levels. No effects were observed on survival, fish condition, and metabolic indices
49 (gonado-, hepato- and spleno- somatic indexes (GSI, HSI, SSI), RNA/DNA and proteins/DNA ratios).
50 Multivariate analyses showed that several biomarkers (DNA damages, micronucleus (MN) frequency,
51 brain acetylcholinesterase (AChE), lysosomal membrane integrity (LMI), phagocytosis activity and

52 reactive oxygen species (ROS) production) were exclusively correlated with fish tritium internal dose
53 rate, showing that tritium induced genotoxicity, as well as neural and immune responses. The results
54 were compared with another study on the same fish species where fish were exposed to tritium and
55 other contaminants in natural environments. Together with the field study, the present work provides
56 useful data to identify biomarkers for tritium exposure and better understand modes of action of
57 tritium on the fathead minnow.

58

59 **Keywords**

60 Fathead minnow, *Pimephales promelas*; tritium internal dose rate; *in vivo* exposure; genotoxicity;
61 immune system response; oxidative stress response; neural response; fatty acid composition

62

63 **Introduction**

64 Tritium (^3H) is a radioactive isotope of hydrogen. Naturally, the main source of tritium is from cosmic
65 rays interacting with nitrogen in the atmosphere. Anthropogenically, it can be produced by the in-
66 reactor neutron activations of lithium, hydrogen and deuterium. Releases of tritium to the environment
67 occur during normal functioning of nuclear power plants (6.0×10^2 to 4.0×10^3 kBq/L in the effluents
68 in France and $<1.5 \times 10^3$ kBq/L in Canada) and nuclear fuel reprocessing plants (10^5 kBq/L in the
69 effluents in France) (HPA 2007, ASN 2010, OPG 2017). In both countries, the average tritium activity
70 concentrations in receiving waters is very low at 1-4 Bq/L (ASN 2010, OPG 2017). The European and
71 Canadian limits of tritium in water for human consumption are 0.1 and 7 kBq/L, respectively (CNSC
72 2008, European_Union 2013). The International Thermonuclear Experimental Reactor (ITER) being
73 built next to the Cadarache facility in France is an international research and engineering reactor due
74 to come into service in 2030. It is expected that tritium releases to water and air will increase after this
75 reactor is in operation (ASN 2010). In the environment, tritium is often found in the form of tritiated
76 water (HTO), which can be incorporated into organic molecules, forming organically bound tritium
77 (OBT) (Bogen et al. 1979). With the anticipated increase in tritium releases, there is a need to better
78 understand tritium effects and particularly its toxic modes of action (ASN 2010).

79 Tritium is a low energy β emitter with a half-life of 12.3 years, which causes high ionization density
80 within a relatively small volume of matter (ASN 2010). Therefore, if enough tritium is ingested, the
81 energy deposited could lead to health consequences. The toxicity of HTO has been studied on aquatic
82 invertebrates and vertebrates (Adam-Guillermin et al. 2012). However, most of the studies reported
83 acute toxicity effects using high dose rates (Real et al. 2004). Studies of the effects of tritium on fish
84 have focused mostly on life-history endpoints linked to reproduction and development (Ichikawa
85 &Suyama 1974, Hyodo-Taguchi &Etoh 1993, Sazykina &Kryshev 2003). Therefore, there is a lack of
86 data on biological effects induced on other biological scales and from chronic low levels of exposure,
87 especially for aquatic vertebrates such as fish (Adam-Guillermin et al. 2012).

88 Chronic low levels of ionizing radiation exposure are not necessarily detrimental. In fact, they were
89 previously shown to induce adaptive responses in fish and amphibians (Smith &Moccia 2001, O'Neill-

90 Mehlenbacher et al. 2007, Audette-Stuart et al. 2011, Audette-Stuart & Yankovich 2011, Mothersill et
91 al. 2014).

92 Tritium exposure has been shown to cause DNA breaks in aquatic invertebrates such as the marine
93 mussel, *Mytilus edulis* (Hagger et al. 2005, Jha et al. 2005, 2006, Jaeschke et al. 2011), and in
94 vertebrates including medaka (Suyama et al. 1981), fathead minnow (Gagnaire et al. 2017) and fish
95 cells (Stuart et al. 2016). Molecular changes including DNA lesions, measured through the comet
96 assay or micronucleus frequency (MN), a biomarker of chromosome damages, are referred to as
97 genotoxicity markers because they indicate that ionizing radiation can affect cells and, if the DNA is
98 left unrepaired, potentially have an effect on individual health or reproductive fitness. However, other
99 kinds of effects were reported, such as the suppression of immune response in rainbow trout (Strand et
100 al. 1982) as well as hormonal effects (Erickson 1971). Immune parameters such as phagocytosis
101 (Fournier et al. 2000), lysosomal membrane integrity (Bado-Nilles et al. 2013), necrosis and reactive
102 oxygen species production (ROS) (Gagnaire et al. 2014) have also been described as biomarkers that
103 can be modified by several kinds of contaminants and can, therefore, be useful to assess the effects of
104 tritium on aquatic vertebrates. If tritium can induce genotoxicity and immunotoxicity, it can be
105 hypothesized that defense from oxidative stress, detoxification and general metabolism would respond
106 to tritium exposure. Therefore, biomarkers of the antioxidant system (catalase (CAT), superoxide
107 dismutase (SOD), glutathione peroxidase (GPX)) and also of the neural system (acetylcholinesterase
108 (AChE)), which are commonly used in ecotoxicological studies (Sanchez & Porcher 2009), may be
109 modified by tritium. Moreover, the combined use of these biochemical markers, which are sensitive
110 molecular and cellular indicators, and physiological markers (metabolic and somatic indices and fatty
111 acid composition), which can show effects occurring at the individual level, is a promising way to
112 relate responses observed at different levels of biological organization (Kerambrun et al. 2012).

113 The fathead minnow, *Pimephales promelas*, a species of freshwater fish native to Canada, has shown
114 to be tolerant to a wide range of water types. Moreover, this species has been used for toxicity testing
115 since the 1960s (Environment_Canada 2011). In chronic low dose field tests (Gagnaire et al. 2017),
116 fathead minnows were exposed to increasing HTO activity concentrations with cross-contamination
117 with metals. It was found that several biomarkers related to immunity, DNA integrity, neural system

118 and fatty acids presented changes that were correlated to fish internal tritium dose rate (Gagnaire et al.
119 2017). Therefore, this study was followed up with a controlled laboratory study in which fathead
120 minnows chronically exposed to low dose tritium spanning a similar range as that found at the field
121 sites were tested to assess genotoxic, immune and neurological responses. Two forms of tritium (HTO
122 and OBT) were assessed because tritium toxicity may also vary as a function of its chemical form
123 (Kim et al. 2015). Minnows were housed in aquaria and exposed over 60 days to water with a range of
124 HTO activity concentrations. Fish were given either non-tritiated feed or feed containing tritiated
125 amino acids. After this exposure period, all fish were maintained in non-tritiated water and given non-
126 tritiated food for another 60 day depuration period. Throughout the study, tritium internalization and
127 biomarkers for genotoxicity, oxidative stress, immunity and fitness were evaluated.

128

129 **Material and Methods**

130 Fish exposure

131 The laboratory study took place between September 2014 and January 2015. Approximately five-
132 month old male fathead minnows (3.93 ± 0.13 g, 6.00 ± 0.08 cm) were received from Aquatics
133 Research Organisms Inc. (Hampton, NH) and progressively allowed to adapt to the reconstituted
134 water. During the study, the fish were kept in reconstituted water prepared by adding 11.0 g of calcium
135 chloride (CaCl_2), 8.4 g of sodium bicarbonate (NaHCO_3), 3.0 g of magnesium sulfate (MgSO_4), 0.4 g
136 of potassium chloride (KCl) and 0.1 g of sodium bromide (NaBr) to 100 L of deionized (reverse
137 osmosis) water. The fish were housed in adjacent rooms that were maintained at $21 \pm 1^\circ\text{C}$. The
138 temperature in the tanks was $19.8 \pm 0.7^\circ\text{C}$ due to frequent water changes; however, all treatment groups
139 including controls were on the same water change schedule, so the potential effects of temperature
140 changes were identical for all groups. Each 40 L or 20 L aquaria was equipped with circulation and
141 filtration systems. Prior to being exposed to tritium, the fish were acclimated to the tank environment
142 for 21 days, after which HTO was added to cover an activity concentration range from <100 Bq/L up
143 to a maximum of 180,000 Bq/L. The aquaria were referred to as C (control), 12K, 25K and 180K to
144 reflect the nominal HTO tritium activity concentrations of 12, 25 and 180 kBq/L. The control aquaria
145 were separated from the test aquaria in a different ventilated room to minimize cross-contamination.
146 To assess the effects of HTO through water and OBT through food on their own and combined, there
147 were three different test conditions (HTO, OBT and a combination of the two), as previously used
148 (Gagnaire et al. 2017). To measure the effects of only OBT, fish in dedicated tanks were fed
149 commercial fish pellets spiked with tritiated amino acids (activity concentration of 27.8 ± 5.7 kBq/L)
150 and the aquaria were filled with non-tritiated water (OBT). The HTO test group received non-tritiated
151 commercial fish pellets, but were housed in water at activity concentrations of either 12, 25 or 180
152 kBq/L. Finally, the third test group combined OBT and HTO, the fish were fed tritiated feed to an
153 activity concentration of 27.8 ± 5.7 kBq/L and the tanks contained water at 25 kBq/L of HTO
154 (25KOBT).
155 The tritium exposure period was for 60 days, after which time the tritium activity concentrations
156 returned to background values (<100 Bq/L) for an additional 60-day depuration period in all tanks.

157 Fish then received non-tritiated feed. The tank water was changed weekly and the fish were fed daily
158 by adding an amount of commercial feed to achieve up to 1% of the total fish biomass in each tank.
159 Water quality parameters for pH, nitrogen compounds, temperature and hardness were regularly
160 measured throughout the study. Fish mortality was recorded. Ten individual fish were randomly
161 sampled from each exposure group for analysis of tritium, physical and biological markers at 60 days
162 and at 120 days. In addition, 10 fish were measured (fork and standard lengths), the fish and their
163 organs were weighed and a blood smear was prepared (to evaluate MN frequency) at day 0, 15, 30, 60,
164 75, 90 and 120, and tritium was measured in their tissues.

165

166 Physico-chemical parameters

167 *HTO measurements*

168 HTO activity concentrations within the aquaria were measured weekly throughout the experiment.
169 Water samples were collected from each aquarium and measured using Ultima Gold scintillation
170 cocktail as previously described (Gagnaire et al. 2017). A standard of 145 Bq/mg tritium was used to
171 determine counter efficiency. Blank samples were also added. The detection limit was 50 Bq/L.

172

173 *Preparation of tritiated feed*

174 Fish were fed with Silver Cup 1.0 mm pellet fish feed from Martin Mills Inc. (Elmira, ON). The OBT-
175 spiked feed was prepared as previously described (Gagnaire et al. 2017). Non-tritiated control food
176 was also prepared in the same way so that fish received the same food quality. The feed was analyzed
177 for tritium and an average value of 27.8 +/- 5.7 kBq.L OBT was obtained.

178

179 *TFWT and OBT measurements and estimated dose rate in fish samples*

180 Ten minnows per treatment group were randomly collected for the analysis of tissue free-water tritium
181 (TFWT) and OBT activity concentrations on days 0, 15, 30, 60, 75, 90 and 120. Carcasses were also
182 pooled (composite samples of 2-4 fish), and analyzed at days 0, 60 and 120.

183 For TFWT measurements, frozen fresh samples were used as previously described (Gagnaire et al.
184 2017). The samples were counted for 100 minutes on a Tri-Carb 3110 TR LSC. The dilution factor

185 was taken into account when the activity concentrations were calculated. The protocol for OBT
186 measurement in animals is described in Kim et al. (2015) and Gagnaire et al. (2017). Samples of non-
187 tritiated Silver Cup feed were also prepared as described in Gagnaire et al. (2017).

188 Estimated internal dose rates were calculated from fish internal activity concentrations of TFWT and
189 OBT using an internal dose coefficient (DC) of 5.38×10^{-3} $\mu\text{Gy/h}$ per Bq/g (EDEN software v2)
190 (Beaugelin-Seiller 2016, Paquet & Harrison 2018) under the hypothesis of a uniform repartition of
191 tritium in tissues and using a radiation weighting factor of 3 in order to consider the relative biological
192 effectiveness of tritium beta emissions compared to gamma radiation (Hunt et al. 2009), as previously
193 described (Gagnaire et al. 2017). The weighting factor of 3 has been debated in the literature, but we
194 chose to follow the same methodology as in the field experiment (Gagnaire et al. 2017) to allow for a
195 direct comparison of results.

196

197 Biological parameters

198 *Fish dissection and sample collection*

199 At several time points (D0, D60, and D120), ten fathead minnows per treatment group were randomly
200 collected for analysis. For D0, 10 fish were analyzed before the beginning of the experiment.

201 Fathead minnows were measured for length, weighed and dissected for endpoint measurements. Fish
202 were euthanized via rapid cooling followed by decapitation according to an animal care protocol
203 approved by the Chalk River Laboratories (CRL) Animal Care Committee. Gonado-Somatic (GSI),
204 Hepato-Somatic (HSI), Spleno-Somatic (SSI) indexes and Fulton's condition factor (K) were
205 calculated as previously described (Amara et al. 2009, Gagnaire et al. 2017). Gender was confirmed
206 during dissection.

207 Blood was used for micronuclei (MN) measurements. The brain, the muscle and the liver were used
208 for enzymatic measurements. Other parts of the muscle and the liver were used for fatty acid
209 composition analysis. Cell viability, phagocytosis, ROS levels and lysosomal membrane stability were
210 assessed in spleen by flow cytometry within 24 hours of collection. Gonad samples were prepared,
211 kept at 4°C overnight, and then used to conduct the viability and comet assay; another part of gonad

212 samples was used to perform the RNA/DNA and protein/DNA determinations. Details are provided in
213 Gagnaire et al. (2017).

214

215 *Biological analyses*

216 Cellular mortality, lysosomal membrane integrity (LMI), ROS basal and phorbol myristate acetate
217 (PMA)-activated levels, and phagocytosis activity were determined using flow cytometry according to
218 protocols described in Gagnaire et al. (2015a, 2017). The comet assay and the micronucleus frequency
219 assay were performed as previously described (Festarini et al. 2016, Gagnaire et al. 2017). For
220 enzymatic assays, brain, liver and muscle samples were homogenized immediately after sampling and
221 the S9 fraction was frozen until analyses. All of the protocols for measuring hepatic GPX, CAT and
222 SOD and brain and muscle AChE are presented in details in Gagnaire et al. (2017). Proteins, RNA and
223 DNA quantification were performed on gonads as previously described (Gagnaire et al. 2017).
224 Samples for fatty acids analysis were sent to Lipid Analytical Laboratories (Guelph, Ontario, Canada).

225

226 Statistical analyses

227 Results are presented as mean \pm standard error (se). For the first raw analyses, normality of data was
228 checked using the Shapiro–Wilk test and when not normal, data were Box-Cox transformed.
229 Differences between conditions were tested with t-tests or ANOVA when the data were normal and
230 with Kruskal-Wallis or Mann-Whitney Rank Sum tests when data were not normal. Analyses were
231 performed using the STATISTICA Software version 12 (StatSoft, Inc., Tulsa, OK, USA), with
232 significance at $p < 0.05$.

233 All multivariate analyses were computed using the R software (version 3.4.3) (R_Core_Team 2017)
234 and RStudio environment (version 1.1.442) (RStudio_Team 2015). Simple linear regressions were
235 used at D60 and D120 in order to assess the individual evolution of each biomarker as a function of
236 increasing tritium exposure. Tritium exposure was characterized by a unique value for each condition
237 (Control, 12K, 25K, OBT, 25KOBT, 180K). These values were the median of internal dose rates
238 measured on individual fish (n comprised between 7 and 16). As the medians were highly dispersed
239 (0.000258 to 0.65 $\mu\text{Gy/h}$), they were log₁₀ transformed in order to be used in regression models.

240 Before performing the regression models, the linearity hypothesis was visually evaluated for each
241 biomarker using scatter plots of type “biomarker vs log10 of internal dose rate median” using the
242 *ggplot2* package. This was satisfied for all comparisons. Finally, the significance of the linear
243 relationship was evaluated using a permutation test using the *lmp* function of the *lmp* package.
244

245 **Results**

246 Physico-chemical parameters

247 *HTO in water and TFWT in fish*

248 At D15 and D30, we saw that TFWT values were consistently the same as the tank water HTO activity
249 concentrations. At D60, however, the fish were transported, from the aquaria to the dissection room, in
250 background water instead of tank water. For those fish, the measured TFWT were much lower because
251 of the fast depuration rate. For dose calculation of the D60 fish, the TFWT was assumed to be the
252 same as HTO of the tank water at the time of sampling.

253 Measured HTO activity concentrations in water are presented in Table 1. Average measured values
254 were 63, 127, 10058, 21629, 20645 and 154314 Bq/L for control, OBT, 12K, 25K, 25KOBT and
255 180K tanks, respectively. The OBT activity concentration in tritiated feed was 27.8 ± 5.7 Bq/L for
256 both the OBT and 25KOBT conditions.

257

258 *TFWT, OBT and estimated dose rate in fish*

259 TFWT and OBT internal concentrations and estimated internal dose rates for all conditions and all
260 time points are presented in Table 1. TFWT internal values followed the HTO activity concentrations
261 in water (Control<OBT<12K<25KOBT=25K<180K). OBT internal concentrations were 24 to 50
262 times lower than TFWT internal concentrations at D60 (except for the OBT condition) and followed a
263 different gradient (Control<12K<25K<OBT<25KOBT<180K). At D120, TFWT internal
264 concentrations had long returned to background level. Note that for the OBT condition, TFWT stayed
265 at background level throughout the study. At D120, OBT internal concentrations were 2 times lower
266 than at D60 and were 1.9 to 17 times higher than TFWT internal concentrations.

267 OBT internal concentrations in 25KOBT compared to 25K were 2.4 times higher at D60 and 1.5 times
268 higher at D120 (Table 1).

269 The highest value of internal dose rate was 0.65 μ Gy/h, obtained for 180K fish at D60 (Table 1).

270 The OBT formation and uptake was studied in fish exposed in the conditions 25K, OBT and 25KOBT
271 (Figure 1). The contribution of water and food to the OBT buildup was investigated over a period of

272 60 days. It was noted that in the early stage of the exposure period (15 days), OBT formation (from
273 water) was the major contributor to the buildup of OBT (Figure 1). Towards the end of the exposure
274 period, however, OBT uptake (from food) was predominating (Figure 1). In contrast, the rate of loss of
275 OBT in fish tissues over a 60 day depuration period showed a similar pattern regardless of the source
276 of the buildup (Figure 1).

277

278 Biological parameters

279 *Survival*

280 The mortality rates were at a maximum of 4% between days 0 and 60, and 2% between days 60 and
281 120, without any differences between groups (data not shown).

282

283 Physiological responses

284 Results were analyzed in the context of researching effects of different conditions and type of
285 exposure on biomarkers.

286

287 *Biological indices*

288 None of the evaluated biological indices (K, HSI, SSI, GSI) showed any significant differences among
289 groups for all time points studied.

290 Values were significantly higher at D60 compared to D120 for K (t test, $t_1=11.7$, $p=5 \times 10^{-21}$). The fish
291 weight to length ratio change for the last time point was not associated with a tritium exposure.

292

293 *RNA/DNA and proteins/DNA*

294 Analyses were performed only at D60. No significant differences among groups were shown for the
295 different parameters.

296

297 *Fatty acids using C18:1 trans and SFA/MUFA as markers*

298 At D60, no differences were shown for parameters measured in the liver and for C18:1 trans in the
299 muscle. The ratio SFA/MUFA in the muscle showed significant differences, with values for controls

300 being lower than values for the 25KOBT group (Kruskal Wallis test, $H_5=15.4$, $p=0.008$) (Figure 2).
301 Neither of the measured parameters presented differences among groups at D120. Values were
302 significantly higher at D60 compared to D120 for SFA/MUFA in liver (t test, $t_1=2.05$, $p= 0.042$).

303

304 Genotoxic responses

305 *Comet assay*

306 At D60, both 25KOBT and 180K groups presented significantly higher DNA damages compared to
307 the control and OBT groups (Figure 3). At D120, a tendency for increased DNA damages was noted,
308 with values higher for the OBT, the 25KOBT and the 180K groups as compared to the 12K and the
309 25K groups, and values for both these groups were higher than for the control group (Figure 3).
310 Values were significantly higher at D60 compared to D120 (Mann-Whitney test U, $U_1=826$, $p= 0.011$).

311

312 *Micronucleus frequency*

313 At D60, the 25KOBT and the 180K groups presented significantly higher MN frequencies than all
314 other groups (Figure 4). At D120, no differences between groups were found (Figure 4). Values were
315 higher at D60 compared to D120 (t test, $t_1=2.17$, $p= 0.03$).

316

317 Immune, oxidative stress and neurotoxic responses

318 Cell mortality presented no significant variations at either D60 or D120. LMI presented significant
319 variations at D120: values were lower in the 12K group compared to the 180K group (Figure 5). At
320 D60, the differences were not significant ($p=0.09$), but a trend of higher values for the OBT, 25KOBT
321 and 180K groups as compared to the controls appeared. Values were higher at D60 compared to D120
322 (t test, $t_1=4.7$, $p= 8 \times 10^{-6}$).

323 The ROS production index presented no significant differences at D60. However, at D60, both basal
324 and activated ROS levels were significantly lower in controls than in the 25KOBT and the 180K
325 groups (Kruskal Wallis test, $H_5= 22.1$ and 19.9 , respectively; $p=0.001$). At D120, the ROS production
326 index values were significantly lower in the 180K group compared to the control and the 12K groups

327 (one way ANOVA, $F_1=3.28$, $p=0.012$) (Figure 6). At D120, results for basal and activated ROS levels
328 were globally the same as D60 (Kruskal Wallis test, $H_5= 22.6$ and 20.8 , respectively; $p=0.001$).
329 Phagocytosis activity presented no significant differences at D60. At D120, values were significantly
330 higher in the 180K group compared to controls (Figure 7).
331 None of the evaluated enzymatic activities (AChE in brain and muscle, SOD and GPX in brain and
332 liver, CAT in liver) showed any significant differences among groups for all time points studied.
333 Values were significantly higher at D60 compared to D120 for muscle AChE (t test, $t_1=6.9$,
334 $p=3.3 \times 10^{-10}$), and lower at D60 compared to D120 for brain AChE, GPX and SOD (t test,
335 $t_1=-4.32$, -12.2 and -5.9 , respectively; $p=4.7 \times 10^{-5}$, 2.8×10^{-12} and 8.7×10^{-8} , respectively).

336

337 *Multivariate analyses*

338 The General Linear Models (GLM) allowed for the evaluation of the potential effects of tritium
339 radiological dose rate in fish tissues.

340 The model showed that several parameters were positively or negatively correlated with fish tritium
341 dose rate (Table 2). At D60, DNA damages ($F_1=0.073$, $p=2 \times 10^{-16}$) and MN frequency ($F_1=1.54$,
342 $p=10^{-20}$) were positively correlated to dose rate. At D120, several parameters were positively
343 correlated to tritium dose rate in fish tissues: DNA damages ($F_1=0.11$, $p=10^{-20}$), brain AChE activity
344 ($F_1=0.42$, $p=0.0224$), spleen LMI ($F_1=84.5$, $p=0.0144$), spleen phagocytosis activity ($F_1=7.41$,
345 $p=0.0014$), spleen ROS activated and basal levels ($F_1=377.8$ and 387.1 , $p=0.0026$ and 0.0002 ,
346 respectively), whereas ROS index was negatively correlated ($F_1=-0.32$, $p=0.0158$) (Table 2).

347

348 **Discussion**

349 *TFWT and OBT in fish tissues*

350 As for the field study (Gagnaire et al. 2017), the increasing gradient of tritium activity concentration in
351 the water was also found in fish tissues for TFWT and OBT at D60. After the beginning of depuration,
352 when tritium was absent from the water, fish tissue TFWT was also rapidly depurated; however, OBT
353 was still present in fish tissues even after 60 days of depuration (D120).

354 As previously discussed (Gagnaire et al. 2017), TFWT levels in fish tissues are assumed to match
355 water levels, with theoretical concentration factors close to 1 (Melintescu et al. 2011). In our study, we
356 saw equilibrium between TFWT and tank water HTO at D15 and D30. At those time points,
357 concentration factors were between 0.76 and 0.81, which is higher than the concentration factors
358 obtained for the field study (Gagnaire et al. 2017). Moreover, exchangeable OBT is supposed to
359 equilibrate with internalized TFWT, leading to an OBT/TFWT ratio close to 1 in aquatic organisms at
360 equilibrium (Melintescu et al. 2011) and in fish (Smith et al. 2006). This was not the case in the
361 present study. As for the field study (Gagnaire et al. 2017), this was expected as it takes time for OBT
362 to accumulate in fish tissues and it also takes a long time for it to depurate (Kim et al. 2015). However,
363 we used our data to evaluate HTO transfer and transformation of internalized TFWT in OBT because
364 this is currently poorly documented (Melintescu & Galeriu 2011).

365

366 *Origin of OBT accumulated in fish tissues*

367 OBT in fish can be formed from two different sources: water and food. To differentiate the sources we
368 have used the terms formation and uptake. Firstly, formation is defined as the buildup of OBT through
369 surrounding water by metabolism. Secondly, uptake is defined as the buildup of OBT through
370 ingestion of OBT contained in the food.

371 Our results indicated that the food chain will be the main contributor to OBT buildup in fish under
372 long-term exposure, such as natural environmental conditions around nuclear facilities. Once fish are
373 no longer exposed to tritium, the depuration time for OBT would be expected to be similar regardless
374 of the form of tritium exposure.

375 A series of trout experiments conducted over 140 days, when fish were contaminated with HTO and/or
376 OBT, reported that OBT was building up considerably faster in tissues when fish were receiving OBT
377 spiked feed (assuming 25% assimilation) compared to being exposed to the same HTO level in their
378 tank water (Kim et al. 2013, Kim et al. 2015). This means that OBT uptake was a greater contributor
379 compared to OBT formation. The rate of OBT build up was also found to change with the fish growth
380 rate, with faster growth rates resulting in lower OBT activity concentration in tissues.

381 In the current study, the OBT activity in the feed represented a smaller fraction than the HTO in the
382 water compared to the trout study. If the exposure conditions were mirrored, for a water HTO activity
383 concentration of about 25 kBq/L, fish would receive OBT in the feed of about 100 kBq/L (again
384 assuming 25% of assimilation). In this study, however, for a HTO activity concentration of about 25
385 kBq/L, the fish were receiving feed at about 27 kBq/L, which corresponds to about 7 kBq/L if 25% of
386 assimilation is assumed. Despite this difference in study design, the results obtained in the current
387 study agree well with the trout study results in the sense that, for chronic exposure, OBT contaminated
388 feed also plays a predominant role in OBT buildup in the tissue.

389

390 *Effects on biological parameters*

391 No significant differences among groups for any of the assessed biological indices were found.
392 Similarly, no effect of tritium was shown for either the RNA/DNA or proteins/DNA ratios in gonads
393 and kidneys. Similar results were obtained after exposure of younger fathead minnows to tritium and
394 other pollutants in a field experiment (Gagnaire et al. 2017). As several of these parameters are a direct
395 or indirect indication of the metabolic growth rate of the organism (Amara et al. 2009), we could
396 hypothesize that the dose rates were too low or that the duration of the experiment was not long
397 enough to see an impact on these integrative indices.

398 In the present study, SFA/MUFA ratio in the muscle was not correlated to fish tritium dose rate. In the
399 field study, the same ratio in liver and also C18:1 trans in muscle were positively correlated to fish
400 tritium dose rate (Gagnaire et al. 2017). Ionizing radiation exposure was shown to modulate fatty acid
401 composition, and the changes are known to be tissue-specific and influenced by diet (Nawar 1973,
402 Audette-Stuart et al. 2012). In the field study, the dietary fatty acids intake differed from the laboratory

403 study because the fish were feeding on organic matter suspended in the water column in addition to
404 the provided feed. The differences noted between the field and the laboratory study may, therefore,
405 reflect differences in diet. Our laboratory findings did not confirm laboratory results obtained in trout
406 exposed to HTO where a decrease of SFA/MUFA ratio was observed in liver and muscle (Festarini et
407 al. 2016). Muscle SFA/MUFA ratio cannot be considered as a direct indicator of tritium effects in fish.
408 The genotoxic biomarkers evaluated in this study presented significant differences in tritium exposed
409 fish: DNA damages measured by the comet assay were positively correlated to tritium internal dose
410 rates for both sampling times. The same result was obtained for MN frequency but only at D60. This
411 result showed that the effect on MN frequency was reversible as they returned to background values at
412 the end of the depuration period. These results suggest that OBT remaining in the tissues at D120 can
413 induce DNA strand breaks in the gonad, but not MN frequency in the blood. However, it should be
414 noted that while sperm cells turnover is really quick (a few days), the turnover of fish erythrocytes is
415 longer (100-400 days) (Soldatov 2005). Damages on blood cells could therefore have been repaired at
416 D120. The same results were obtained in the field experiment, with a reversible effect on MN
417 frequency and non-reversible effects, within 60 days of depuration, on DNA damages (Gagnaire et al.
418 2017). Moreover, in the field study, both of these parameters were positively correlated with fish
419 tritium dose rate (Gagnaire et al. 2017); in the present lab study, the same result was obtained for MN
420 frequency at D60. The effects observed on these genotoxic biomarkers can therefore be related to
421 tritium.

422 Tritium was previously shown to increase MN frequency and DNA damages in adult mussels exposed
423 at 12.5 $\mu\text{Gy/h}$ of HTO (Jha et al. 2005, 2006) and 79 $\mu\text{Gy/h}$ of tritiated glycine (Jaeschke et al. 2011),
424 doses 19 and 121 times higher, respectively, than the highest one estimated in our study, showing that
425 fathead minnow is more sensitive to tritium than invertebrates considering DNA damages.
426 Cytogenetic effects were also shown in medaka eggs and embryo-larvae of marine mussel, *Mytilus*
427 *edulis*, exposed to high dose rates of tritium (Suyama et al. 1981, Hagger et al. 2005). DNA damages
428 also increased after exposure of sticklebacks around former uranium mining sites (Le Guernic et al.
429 2016). Increased DNA damages were also shown after exposure of daphnids, *Daphnia magna*, to dose
430 rates of gamma-rays as low as 7 $\mu\text{Gy/h}$ (Parisot et al. 2015). Increased DNA damages were also shown

431 after exposure of zebrafish larvae to 33 $\mu\text{Gy/h}$ of gamma-rays (Gagnaire et al. 2015b) and 400 $\mu\text{Gy/h}$
432 of tritium (Gagnaire et al, unpublished data). At 400 $\mu\text{Gy/h}$, still on zebrafish larvae, the expression of
433 genotoxic markers *h2afx* and *ddb2* involved in DNA repair were both induced in response to HTO
434 exposure (Arcanjo et al., unpublished data). In the same way, DNA damages and MN frequency
435 increased in fish cell lines exposed to tritium (Stuart et al. 2016). Gamma-H2AX was also correlated
436 to tritium dose rate in the field experiment (Gagnaire et al. 2017). These results suggest the
437 establishment of defense against DNA damage by the over-expression of DNA repair genes. Taken all
438 together, these results confirm that radionuclides can induce genotoxicity and that the cells respond by
439 upregulating DNA repair genes.

440 The immune system also responded to tritium exposure. The response was, however, often detected
441 later in the study, mostly after the depuration period. At D120, LMI and phagocytosis activity were
442 higher and ROS production index was lower in the 180K fish. LMI and phagocytosis activity were
443 positively correlated with tritium dose rate in fish tissues at D120, while ROS index was negatively
444 correlated with tritium dose rate in fish tissues. The same increase in LMI was found in the field study
445 at both sampling times (Gagnaire et al. 2017). Even if LMI was shown to be affected by metals (Bado-
446 Nilles et al. 2013), we could hypothesize that the effects observed on LMI were, in part, due to tritium.
447 Contrary to the lab study findings, no statistically significant effects on phagocytosis were shown in
448 the field study; however, this biomarker did correlate with the HTO conditions in the depuration
449 period (Gagnaire et al. 2017). An increase of phagocytosis indicates an enhanced capacity of the
450 organism to protect against infections, but could also lead to an increase of oxidative stress (Whyte
451 2007). Although ROS production was not investigated in the field study, the laboratory study showed
452 that increased phagocytosis did not correlate with an increase in oxidative stress. It has been shown
453 that tritium induced no effect on ROS production index in zebrafish larvae exposed to HTO; however,
454 ROS activated level increased in HTO larvae after 4, 7 and 10 days of exposure to 280-380 $\mu\text{Gy/h}$
455 (Gagnaire et al, unpublished data). The ROS index was also shown to be affected by gamma radiation
456 exposure (Gagnaire et al. 2015b). Tritium has been shown to negatively affect the primary immune
457 response in juveniles of rainbow trout after 20 days of exposure to 83 $\mu\text{Gy/h}$ (Strand et al. 1982),
458 suggesting that the fish immune system seems to be an important target of tritium.

459 In this study, none of the antioxidant parameters (SOD, CAT, GPX) showed any variations with
460 tritium concentrations, but brain AChE was correlated to fish tritium dose rates, as in the field study
461 (Gagnaire et al. 2017). For the field study, SOD and CAT activities were also correlated to fish tritium
462 dose rate, but at different time points depending on the organ (Gagnaire et al. 2017). However, in the
463 field study, metals were also present in the water of the tested sites in addition to tritium. Many studies
464 have shown that alterations of these parameters are mostly caused by metals or pesticides (Sanchez et
465 al. 2005, Richetti et al. 2011). The fact that these activities presented variations in the presence of
466 metals associated with tritium and not with tritium alone leads us to think that these markers are not
467 suitable for the assessment of chronic low level tritium exposure in fish.

468

469 *Relationships between observed effects and tritium internal dose rate*

470 Research on the effects of tritium on fish has mostly addressed reproduction and fecundity. Strand et
471 al. (1982) showed mortality and malformations in trout eggs after 21 days of HTO exposure at 12
472 $\mu\text{Gy/h}$ (total dose of 6 mGy). In medaka, the number of germ cells changed in embryos exposed to
473 7080 $\mu\text{Gy/h}$ for 10 days (total dose of 1.7 Gy) (Etoh &Hyodo-Taguchi 1983), and the number of
474 primary spermatogonia (Hyodo Taguchi &Egami 1977), vertebral malformations and fecundity
475 (Hyodo-Taguchi &Etoh 1986, 1993) were affected at 1200 $\mu\text{Gy/h}$ in adult fish exposed for 30 days
476 (total dose of 864 mGy). In the puffer fish, *Fugu niphobles*, HTO induced a decrease of egg hatching
477 at 12100 $\mu\text{Gy/h}$ following a 130 h exposure period (total dose of 1.54 Gy) (Ichikawa &Suyama 1974).
478 All of these observations were made at much higher levels than the doses studied in the present study.
479 In fact, at D60, the highest dose rate calculated in our study was 0.65 $\mu\text{Gy/h}$, which corresponds to a
480 total dose of 936 μGy .

481 The maximal dose rate studied in the laboratory setting was higher than the highest dose rate
482 calculated in the field study. In the field study, over a 60 day exposure period, the total dose was
483 216 μGy (Gagnaire et al. 2017). Both of these values are considerably lower than the studies reporting
484 effects of tritium on fish. The values of dose rates observed in these field and laboratory studies were
485 also lower than the benchmark used for ecosystem protection of 10 $\mu\text{Gy/h}$ (Garnier-Laplace et al.

486 2010). It follows that the low dose rates and cumulative doses studied were not expected to induce
487 deterministic effects on fathead minnows, and our observations are in agreement with this.

488 The minimum Low Observed Effect Dose Rate (LOEDR) for tritium has been shown to reach
489 0.62 $\mu\text{Gy/h}$ for invertebrate larval development and 12.1 $\mu\text{Gy/h}$ on fish development (Adam-
490 Guillermin et al. 2012). The highest dose rate obtained in this study for 180K fish at D60 was 0.65
491 $\mu\text{Gy/h}$, 18 times lower than the LOEDR for fish development. As for the field study (Gagnaire et al.
492 2017), at this low dose rate, several biomarkers were found to be exclusively modulated by tritium
493 internal dose rate, including blood MN frequency (at D60), gonad comet tail moment, spleen LMI,
494 phagocytosis activity and ROS production, and muscle SFA/MUFA ratio (at D120).

495

496 **Conclusion**

497 The present study aimed to evaluate the effects of HTO and OBT on *P. promelas* in laboratory
498 conditions. Fish health was not affected by tritium exposure as no effects were shown on survival,
499 condition and physiological indices. Considering the low dose rates (maximum of 0.65 $\mu\text{Gy/h}$), this
500 result is not surprising, as the DCRL (Derived Consideration Reference Levels) in which deleterious
501 effects are expected on young fish are supposed to be 4 mGy/h (ICRP 2008). However, at these low
502 dose rates, several biomarkers were correlated with the internal tritium dose rate. Therefore, this study
503 showed that tritium induced genotoxicity, neural and immune responses as we saw modulations of
504 DNA damages, micronucleus frequency, brain AChE, lysosomal membrane integrity and phagocytosis
505 activities. No effects were observed on SOD, CAT, GPX activities and RNA/DNA/protein ratios.

506 In this study, as no effects appeared on health indices, we can conclude that no adverse effect on
507 fitness was shown at tritium concentrations 25 times and 1800 times higher than Canadian and
508 European drinking water limits, respectively. This result indicates nonlinear dose-response
509 relationships for these indices. Effects on these indices may be observed at higher tritium
510 concentrations when the exposure threshold is reached.

511 When comparing our results with those obtained in another study using the same fish species exposed
512 to tritium in natural environments also containing other contaminants, results were similar for several
513 biomarkers: immune (phagocytosis activity, ROS production, lysosomal membrane integrity) and
514 genotoxic parameters (MN frequency and DNA damages). Therefore, the multi-biomarker approach
515 used in both of these studies allowed us to identify the most relevant biological pathways involved in
516 response to tritium exposure in fathead minnows in laboratory and field settings, and consists in an
517 interesting approach for the assessment of radionuclide effects in aquatic ecosystems.

518

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523

524 **Figure Legend**

525

526 **Figure 1:** Relative OBT build up and loss patterns during exposure and depuration periods.

527 **Figure 2:** SFA/MUFA ratio in the muscle of fathead minnows at D60 and D120. Kruskal-Wallis test,
528 $p < 0.01$; $a < b$.

529 **Figure 3:** Results of the comet assay, expressed as mean tail moment, obtained in the gonad of fathead
530 minnows at D60 and D120. Kruskal-Wallis test, $p < 0.0001$; $a < b < c < d$.

531 **Figure 4:** Micronucleus frequency obtained from the blood of fathead minnows sampled at D60 and
532 D120. Two-way ANOVA, $p < 0.0001$; $a < b$.

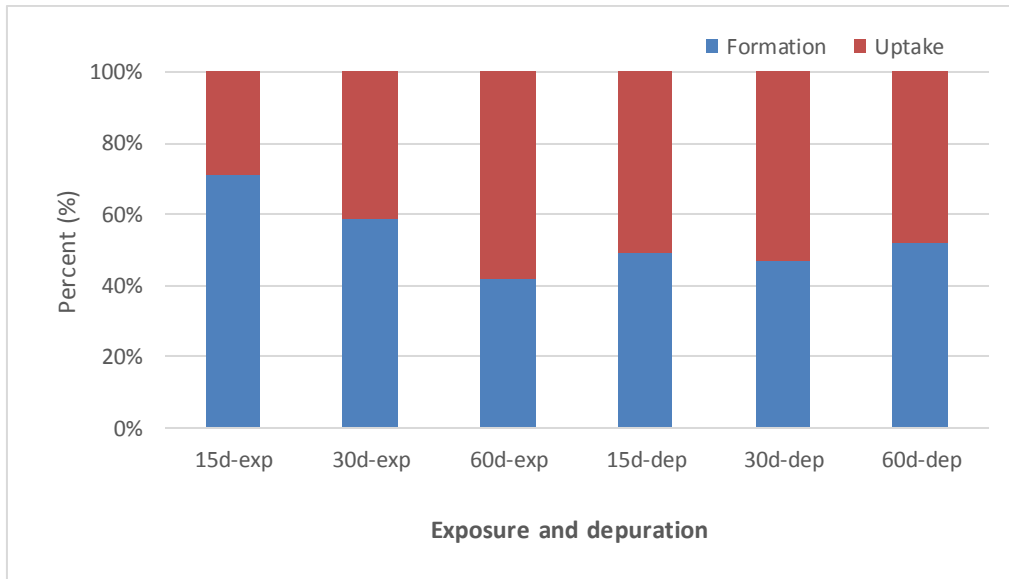
533 **Figure 5:** Lysosomal membrane integrity (LMI) expressed as mean fluorescence intensity (MFI),
534 measured in the spleen of fathead minnows at D60 and D120. Two-way ANOVA, $p < 0.05$; $a < b$.

535 **Figure 6:** ROS index (ratio activated/basal ROS levels expressed as MFI) measured in the spleen of
536 fathead minnows at D60 and D120. Two-way ANOVA, $p < 0.05$; $a < b$.

537 **Figure 7:** Phagocytosis activity expressed as percentage of cells containing three beads or more,
538 measured in the spleen of fathead minnows at D60 and D120. Two-way ANOVA, $p < 0.001$; $a < b$.

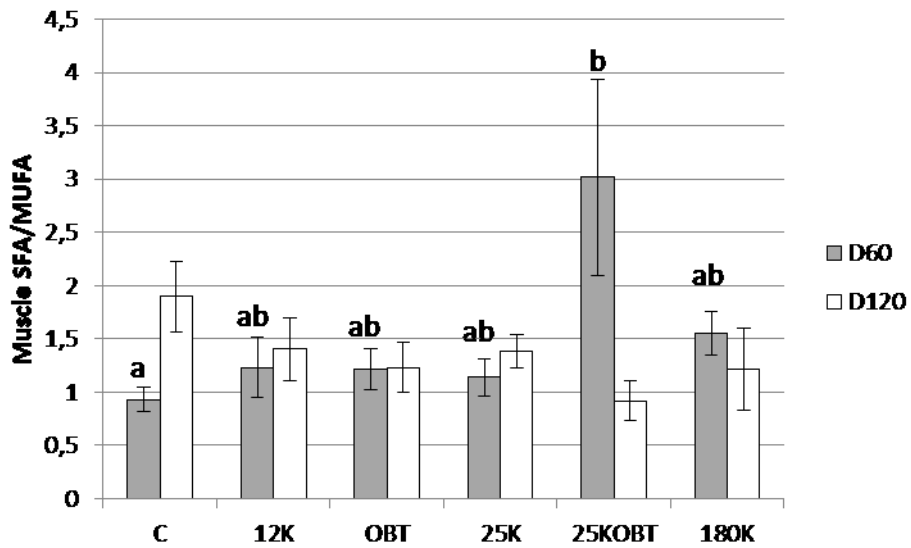
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542 Figure 1



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544 Figure 2

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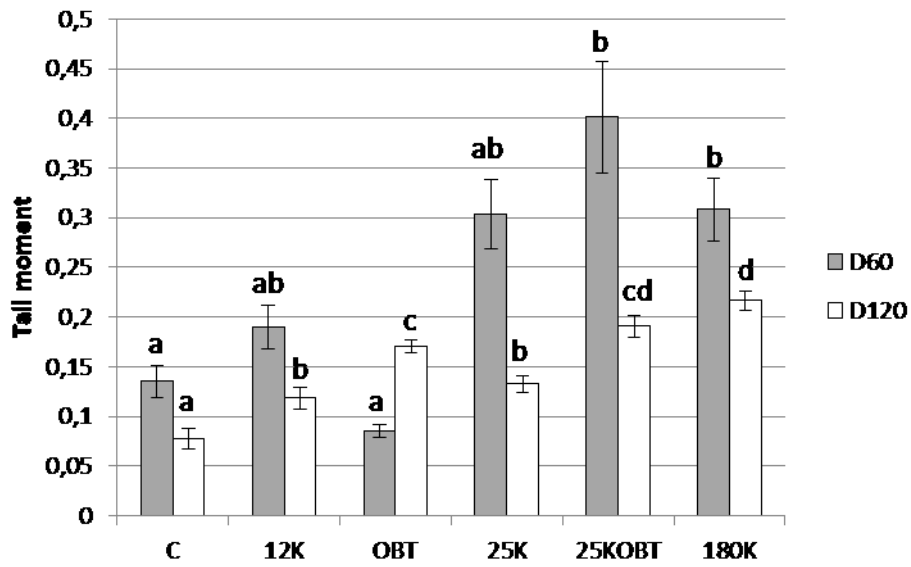
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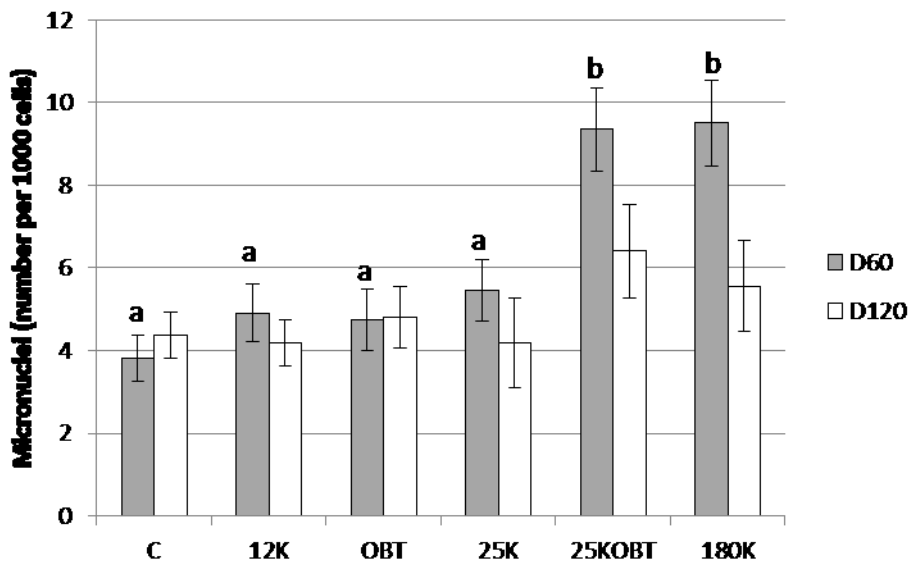
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553 Figure 3

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556 Figure 4

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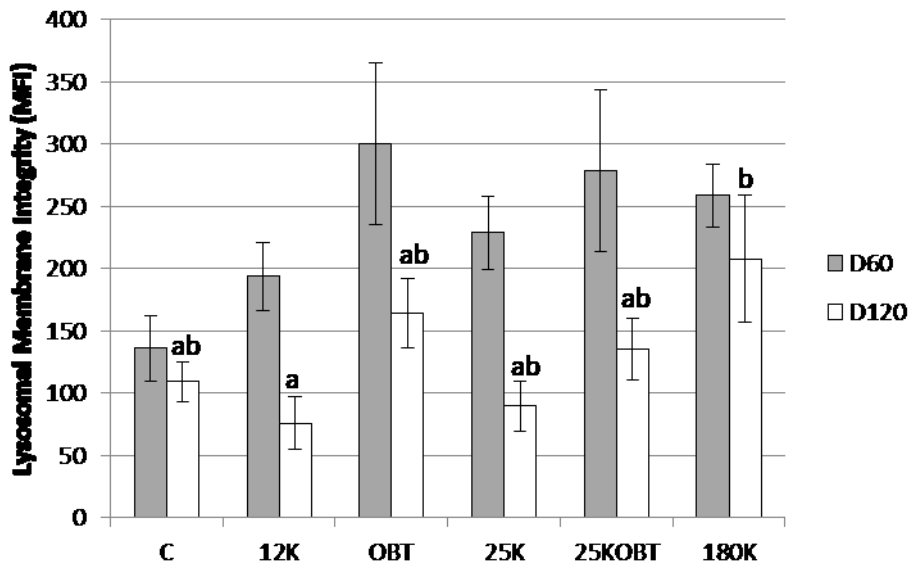
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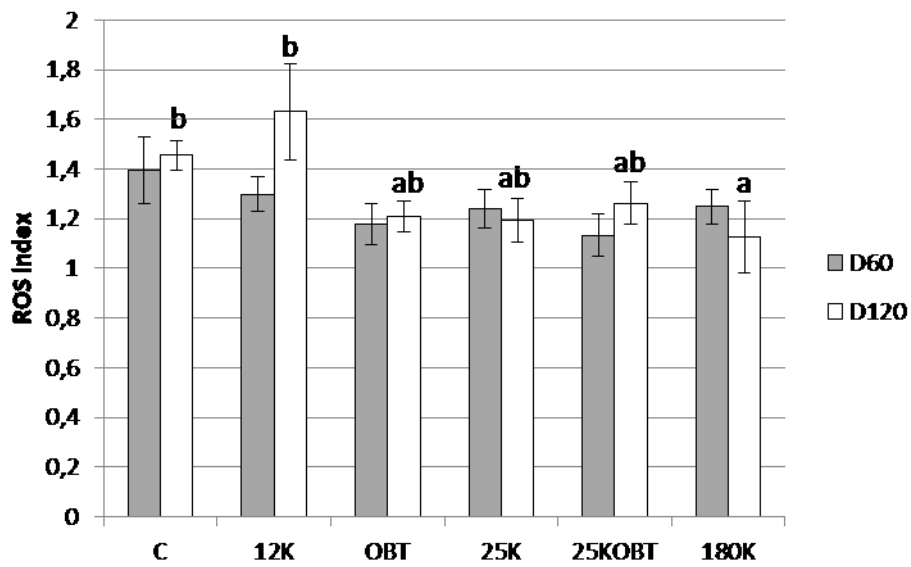
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564 Figure 5

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567 Figure 6

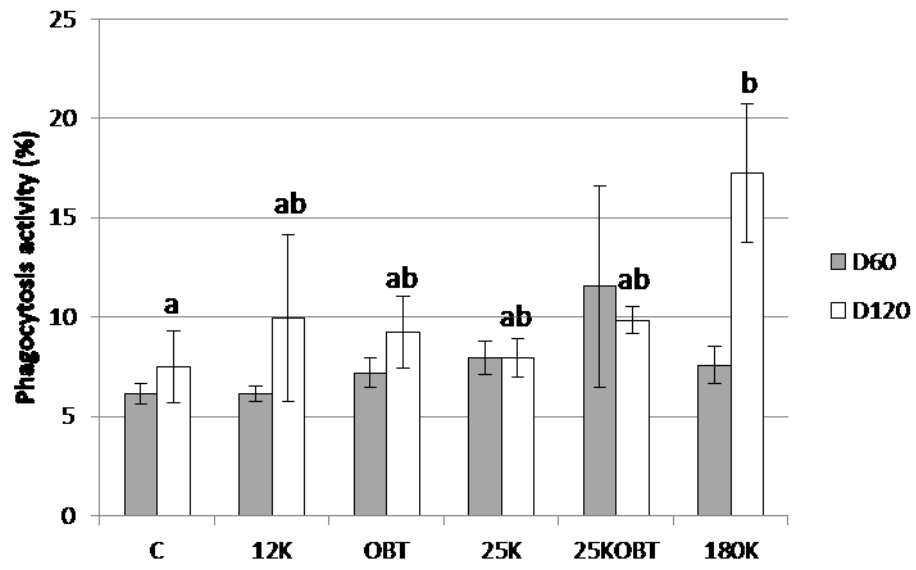
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574 Figure 7

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Table 1: HTO water concentration, HTO and OBT activity concentrations (Bq/kg fresh) and associated estimated dose rate ($\mu\text{Gy/h}$) in fish during the experiment. <dl: less than detection limit. Values presented contain at least ten replicates +/- standard error. The water equivalent factor (WEF) was calculated using our fish water content (Water content=75%, WEF=0.6). At D60, concentration factor was not calculated because as fish were transported in background water instead of tank water, their measured TFWT were much lower because of the fast depuration rate. At D120, HTO water activity concentrations were either below or above DL; therefore, only one value corresponding to the mean of all values obtained is presented.

Time point	Condition	HTO water activity concentration Bq/L	Internal activity concentration			Concentration factor (TFWT fish/HTO water)	Estimated dose rate
			TFWT Bq/kg (fresh)	OBT Bq/kg (fresh)	Total tritium (TWWT+OBT) Bq/kg (fresh)		Total tritium (3H DR) $\mu\text{Gy/h}$
D60	C	63	15 +/- 2	22 +/- 5	42 +/- 6	---	2.3E-04 +/- 3.2E-05
	12K	10058 +/- 1298	8193 +/- 219	192 +/- 13	8396 +/- 227	---	4.5E-02 +/- 1.2E-03
	25K	21629 +/- 612	16477 +/- 135	292 +/- 10	16780 +/- 142	---	9.0E-02 +/- 7.6E-04
	OBT	127 +/- 71	29 +/- 3	419 +/- 20	452 +/- 19	---	2.4E-03 +/- 1.0E-04
	25KOBT	20645 +/- 3033	16748 +/- 669	691 +/- 23	17449 +/- 683	---	9.4E-02 +/- 3.7E-03
	180K	154314 +/- 6239	118713 +/- 1141	2377 +/- 99	121153 +/- 1203	---	6.5E-01 +/- 6.5E-03
D120	C	<100	38 +/- 13	31 +/- 1	69 +/- 13	0.49	3.7E-04 +/- 6.9E-05
	12K	<100	49 +/- 11	94 +/- 5	147 +/- 13	0.63	7.9E-04 +/- 6.9E-05
	25K	<100	35 +/- 8	180 +/- 12	227 +/- 17	0.45	1.2E-03 +/- 9.1E-05
	OBT	<100	19 +/- 2	176 +/- 4	197 +/- 6	0.24	1.1E-03 +/- 3.0E-05
	25KOBT	<100	18 +/- 1	275 +/- 13	301 +/- 11	0.23	1.6E-03 +/- 6.0E-05
	180K	<100	58 +/- 3	990 +/- 52	1046 +/- 52	0.75	5.6E-03 +/- 2.8E-04

Table 2: Results of GLM analyses used to explain the evolution of biomarkers as a function of tritium fish dose rate (3H DR). The table only presents the significant results obtained at D60 and D120. ‘positive’, ‘negative’ indicates the trend of the interaction between the biomarker and the parameter explaining its evolution. $p < 0.05$ for all trends indicated.

Time	Biomarker	3H DR
D60	MN frequency	positive
	DNA damages	positive
D120	DNA damages	positive
	LMI	positive
	Phagocytosis activity	positive
	ROS activated level	positive
	ROS basal level	positive
	ROS index	negative
	Brain AChE	positive

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