

1 Quantitative Analysis of Effects of a Single ^{60}Co Gamma Ray
2 Point Exposure on Time-Dependent Change in Locomotor
3 Activity in Rats

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23

24 **Abstract**

25 Fatigue is one of the earliest nonspecific symptoms of radiation exposure in humans,
26 but its etiology, mechanism, and dose dependency remain unexplained. Investigating initial
27 behavioral changes caused by irradiation of animals might provide important information to
28 aid understanding of early health effects of radiation exposure and clinical features of
29 radiation injury. Although previous studies in rodents suggested that radiation exposure
30 leads to reduced activity, detailed properties of the effects were unrevealed due to a lack of
31 proper statistical analysis, which is needed to better elucidate details of changes in
32 locomotor activity. Ten-week-old male Wistar rats were subjected to single point external
33 whole-body irradiation with ⁶⁰Co gamma rays at 0, 2.0, 3.5, and 5.0 Gy (4 rats per group).

34 Infrared sensors were used to continuously record locomotor activity of each rat. Cumulative
35 number of movements during the night was defined as “activity” for each day. A non-linear
36 mixed effects model accounting for individual differences and daily fluctuation of activity was
37 applied to analyze the rats’ longitudinal locomotor data. Despite a small number of animals
38 per group, our statistical method successfully revealed characteristics of the changes in
39 locomotor activity after radiation exposure, showing that 1) reduction in activity occurred
40 immediately—and in a dose-dependent manner—after irradiation and 2) recovery to
41 pre-irradiation levels required almost one week, with the same recovery rate in each dose
42 group. In addition to improving our understanding of radiation effects on locomotor activity,
43 this statistical framework should be useful to analyze other data with similar structure.

44

45 **1. Introduction**

46 In humans, one of the earliest effects of radiation exposure to the whole body or to a large
47 portion of the whole body is a prodromal period of nonspecific signs and symptoms such as nausea,
48 emesis, fatigue, fever, and anorexia [1–2]. The prodromal syndrome is generally mild or absent at
49 total body doses of 1 Gy or less and occurs from minutes to days following exposure [3–5]. However,
50 it is unclear to what extent these symptoms are psychogenic versus radiation-induced. Therefore,
51 the relationship between initial symptoms and radiation dose is not well understood.

52 Early effects of irradiation have been studied in regard to radiation therapy. In a detailed
53 study of the incidence and severity of side effects during the course of radiation therapy, fatigue was
54 the most prevalent and the most severe symptom reported by patients [6]. With fractionated doses of
55 radiation for cancer treatment, radiation-induced fatigue sets in within a few days after start of
56 treatment and decreases after treatment completion [7]. Although the underlying mechanisms of
57 fatigue have been studied under several disease conditions, an understanding of the etiology,
58 mechanisms, and risk factors of radiation-induced fatigue remains elusive, and this symptom
59 remains poorly managed [8-10]. Investigating initial radiation-related behavioral changes by using
60 animals might provide important information to aid understanding of the health effects of radiation
61 exposure and clinical features of radiation injury.

62 In animals, there have been many studies of radiation-induced behavioral effects, and
63 performance decrement after irradiation has been noted in several reports. A sub-lethal dose of
64 gamma radiation suppressed aggressive behavior in male mice [11], a lethal dose of gamma
65 radiation suppressed locomotor activity in mice [12], and a sub-lethal dose of X-irradiation
66 suppressed volitional activity in rats [13]. Landauer (2002) provided a review of expected
67 performance decrement after radiation exposure [14]. These reports showed that ionizing radiation
68 temporarily suppresses animals' behavior, but that the effect does not continue for a long period.
69 York et al. reported that, 6 h after gamma irradiation with 50 or 200 cGy, spontaneous locomotor

70 activity in mice was 35% or 36% lower, respectively, than in sham irradiated controls, and that their
71 activity recovered to sham irradiated level 12 h after irradiation [15].

72 Although many animal behavioral experiments have a time-dependent data structure with
73 variation among individuals, analyses have typically been performed only at individual time points
74 with no parameterization of the trend in activity over time. Therefore, quantitative analyses have not
75 been made directly on the chronological features. To obtain more detailed and accurate information
76 from data obtained in animal behavior experiments with time-dependent structure and individual
77 variability, application of statistical theory would suggest that analysis based on a mixed effects
78 model [16–17] is both appropriate and effective.

79 The purpose of the present study was therefore to examine in detail the changes over time in
80 locomotor activity of rats immediately after external irradiation with ^{60}Co gamma rays by using such
81 statistical models. Specifically, we aimed to assess the time when reduction of locomotor activity
82 begins, the time when locomotor activity recovers to pre-irradiation level, the dose dependency of the
83 degree of reduction in locomotor activity, and the dose dependency of the rate of recovery. There are
84 individual differences in animal behavior that cannot be ignored, even if the animal type, gender, and
85 weight are uniform. In addition, when animals are observed over a long period of time, it is expected
86 that common changes in behavior will occur due to indoor conditions such as temperature, humidity,
87 and noise, which can change daily, and it is necessary to adjust for these sources of variation.

88 **2. Materials and Methods**

89 **2.1. Experimental Design and Data Collection**

90 **2.1.1. Animals.** The experiment was approved by the Animal Experiment Committee of
91 Semey Medical University, Republic of Kazakhstan, and was conducted in accordance with the
92 Institutional Guide for Animal Care and Use. Ten one-week-old male Wistar rats were purchased
93 from the Kazakh Scientific Center of Quarantine and Zoonotic Diseases, Almaty, Kazakhstan and
94 allowed free access to a basal diet and tap water. Animal rooms were maintained at 19-22 °C with
95 relative humidity 30–70% and a 12 h light cycle. Body weights were measured twice a week during
96 the experiment. At 11 weeks of age, the rats were randomly divided into four groups: control (4 rats)
97 and three irradiated groups (4 rats/group). Each irradiated group received 2, 3.5, or 5.0 Gy of whole
98 body gamma irradiation. Controls were handled with all conditions the same as with the other groups,
99 except that they were not irradiated (dose 0 Gy). The LD₅₀₍₃₀₎ for this strain of Wistar rats is 7 Gy with
100 cobalt-60 radiation [18].

101 **2.1.2. Irradiation with ⁶⁰Co gamma-rays** Irradiation was performed with a Teragam K-2
102 unit (UJP Praha, Praha-Zbraslav, Czech Republic) at the Regional Oncology Dispensary of Semey.
103 Rats were irradiated at 1 m distance from the ⁶⁰Co source at a dose rate of 2.6 Gy/min. Half of the
104 radiation dose was administered from the top and the other half was administered from the bottom. A

105 radiophotoluminescence glass dosimeter, GD-302M [Chiyoda Technol Co., Tokyo, Japan], was used
106 for measuring the doses.

107 **2.1.3. Measurements of daily locomotor activity** Locomotor activities (hereafter
108 abbreviated as “activities”) of the rats were measured with infra-red sensors (Model NS-AS01;
109 Neuroscience, Inc., Tokyo, Japan) placed 16 cm above the open-top cages (26.5 x 43 x 14.5 cm).
110 Numbers of movements were counted on the basis of change in the strength of infra-red rays emitted
111 from the animals. The rats were placed in separate cages, each outfitted with a sensor, and
112 movements were continuously counted by a computerized analysis system (16 channel Multi-digital
113 Counter System [MDC] and DAS System software, Neuroscience, Inc. Tokyo, Japan).
114 Measurements were started 3 days before irradiation and continued for 20 days after irradiation.

115 **2.1.4. Ethical approval** All applicable international, national, and/or institutional guidelines for
116 the care and use of animals were followed. The animal experiment was approved by the Animal
117 Experiment Committee of Semey Medical University, Republic of Kazakhstan (Protocol No 5 dated
118 16.04.2014), and conducted in accordance with the Institutional Guide for Animal Care and Use.

119 **2.2. Statistical analyses**

120 **2.2.1. Definition of daily activity** Because rats are nocturnal animals [19], cumulative
121 number of movements was recorded during the period between 18:00 and 06:00; the number of

122 movements so recorded was defined as activity of a rat in one day. As shown in Fig 1, rates of
123 increase in cumulative movements (slopes) were steeper during nighttime (18:00–05:59) than
124 during daytime (06:00–17:59); i.e., the rats were more active at night, as expected.

125

126 **Fig 1. Cumulative number of movements of each of the 16 rats over a 36-hour period.**

127

128 This suggests that the activity defined in this study represents the nocturnal characteristic of rats and
129 it shows that the measure has relevance as an indicator of a rat's activity.

130 **2.2.2. Data modeling** Logarithmic values of daily activity of each rat as a function of elapsed

131 time relative to day of irradiation are shown for each group in Fig 2.

132

133 **Fig 2. Daily activity of each of four rats belonging to four groups.** The vertical axis shows

134 logarithm of daily activity (number of nocturnal movements) and the horizontal axis shows elapsed

135 time in days relative to the day of irradiation (indicated by arrows): (a) the control group, (b) 2.0 Gy

136 group, (c) 3.5 Gy group, and (d) 5.0 Gy group.

137

138 An acute decrease in activity after irradiation followed by quick recovery to the pre-irradiation level can

139 be seen in every exposed group, whereas no such change or trend was observed in the control

140 group. There also was large inter-animal variation with daily fluctuation in activity. Therefore we
 141 assumed a non-linear mixed effects model [16–17] that takes into account the dose dependency of
 142 the decrease in activity, the dose dependency of the recovery rate, individual differences among
 143 animals, and daily fluctuations within individual animals. For comparison, we fit a simple non-linear
 144 regression model in which individual differences and daily fluctuations were not taken into account.

145 **2.2.3. Non-linear mixed effects model (NLMM)** Let y_{it} be the log transformed
 146 observed activity of rat i at time t in days since irradiation with dose D_i ($t = -3, \dots, 20$; $i = 1, \dots, 16$),
 147 where " $t = 0$ " indicates day of irradiation. We assume the model

$$148 \quad y_{it} = f(t | D_i, \theta) + \delta_i + \eta_t + \varepsilon_{it},$$

$$149 \quad f(t | D_i, \theta) = \xi_0 + \xi_1 t + \xi_2 t^2 - (\beta_1 D_i + \beta_2 D_i^2) \cdot \exp\left[\left\{-\omega_1 \cdot e^{-\omega_2(D_i - D_0)}\right\} t\right] \cdot h(t),$$

$$150 \quad \delta_i \square N(0, \psi^2), \quad \eta_t \square N(0, \varphi^2), \quad \varepsilon_{it} \square N(0, \sigma^2), \quad t = -3, -2, \dots, 20, \quad i = 1, \dots, 16, \quad (1),$$

151 where $\theta = (\xi_0, \xi_1, \xi_2, \beta_1, \beta_2, \omega_1, \omega_2)$ denotes unknown parameters for fixed effects to be
 152 estimated. The term $\xi_0 + \xi_1 t + \xi_2 t^2$ expresses the time dependency of activities without radiation
 153 exposure. The term $\beta_1 D_i + \beta_2 D_i^2$ expresses whether the dose effect in the initial decrease is linear
 154 ($\beta_2 = 0$) or quadratic ($\beta_2 \neq 0$), and the term $-\omega_1 \cdot e^{-\omega_2(D_i - D_0)}$ denotes whether the recovery rate
 155 depends on dose ($\omega_2 \neq 0$) or not ($\omega_2 = 0$). D_0 denotes a fixed pre-assigned dose value for
 156 covariate centering (in this study 2.75 Gy is adopted), $\Delta = (\psi^2, \varphi^2, \sigma^2)$ are unknown dispersion
 157 parameters to be estimated, and the terms δ_i , η_t and ε_{it} represent independent random effects

158 due to individual variability, daily fluctuation, and measurement error, respectively. The function

159 $h(t) : h(t) = 0 (t < 0), h(t) = 1 (t \geq 0)$ denotes the Heaviside function of t to indicate pre- and

160 post-irradiation dichotomy.

161 Let $\mathbf{y} = (\mathbf{y}_1', \dots, \mathbf{y}_{16}')'$, $\mathbf{y}_i = (y_{i,-3}, \dots, y_{i,20})'$, $i = 1, \dots, 16$. It follows from Model (1) that \mathbf{y}

162 has a multivariate normal distribution with mean $\boldsymbol{\mu}(\boldsymbol{\theta}) = (\boldsymbol{\mu}_1(\boldsymbol{\theta})', \dots, \boldsymbol{\mu}_{16}(\boldsymbol{\theta})')'$, $\boldsymbol{\mu}_i(\boldsymbol{\theta}) = f(\mathbf{t} | D_i, \theta)$,

163 $\mathbf{t} = (-3, -2, \dots, 20)'$, $i = 1, \dots, 16$, and variance-covariance matrix

164 $\boldsymbol{\Omega}(\boldsymbol{\Delta}) = I_{16} \otimes (\rho^2 J_{41} + \sigma^2 I_{41}) + J_{16} \otimes \psi^2 I_{41}$, where I_m denotes an m -dimensional unit matrix, and

165 $J_m = \mathbf{1}_m \otimes \mathbf{1}_m'$. Then the likelihood function of $(\boldsymbol{\theta}, \boldsymbol{\Delta})$ can be expressed as

166 $L(\boldsymbol{\theta}, \boldsymbol{\Delta}) = \frac{1}{(2\pi)^8 \sqrt{|\boldsymbol{\Omega}(\boldsymbol{\Delta})|}} \exp\left(-\frac{1}{2} \{\mathbf{y} - \boldsymbol{\mu}(\boldsymbol{\theta})\}' \boldsymbol{\Omega}(\boldsymbol{\Delta})^{-1} \{\mathbf{y} - \boldsymbol{\mu}(\boldsymbol{\theta})\}\right)$. Therefore, the maximum likelihood

167 estimates of $(\boldsymbol{\theta}, \boldsymbol{\Delta})$, denoted by $(\hat{\boldsymbol{\theta}}, \hat{\boldsymbol{\Delta}})$, are obtained by minimizing the quantity

168 $Q(\boldsymbol{\theta}, \boldsymbol{\Delta}) = \log(|\boldsymbol{\Omega}(\boldsymbol{\Delta})|) + \{\mathbf{y} - \boldsymbol{\mu}(\boldsymbol{\theta})\}' \boldsymbol{\Omega}(\boldsymbol{\Delta})^{-1} \{\mathbf{y} - \boldsymbol{\mu}(\boldsymbol{\theta})\} + 16 \times \log(2\pi)$. When $\psi^2 = \varphi^2 = 0$, Model (1)

169 reduces to an ordinary non-linear regression model (NLRM).

170 **2.2.4. Algorithm and software for implementation of data analyses** The

171 unknown parameters were estimated by using an algorithm for optimization with the

172 limited-memory version of the Broyden–Fletcher–Goldfarb–Shanno method [20] to maximize the

173 likelihood derived from the model (1), and the AIC (Akaike Information Criterion) [21] and BIC

174 (Bayesian information criterion) [22–23] were calculated. The function ‘optim’ in the R software ver.
175 3.5.1 was used for carrying out numerical analyses.

176 Maximum likelihood (ML) or restricted maximum likelihood (REML) [24] estimates of the
177 parameters in the linear mixed-effects models can be computed with the “lmer” function in the
178 “lme4” package for R [25]. In this study, the ML method was used to compare the goodness-of-fit
179 of models with the AIC criterion. Estimation results were almost the same with both methods.

180 **3. Results**

181 **3.1. Result of Regression Analysis**

182 **3.1.1. Estimation of fixed effect parameters.** Regression analysis was first
183 performed with all parameters of the NLMM (full NLMM), then model selection was applied by
184 choosing the smallest AIC to determine the optimal NLMM (optimal NLMM). The full NLRM and
185 optimal NLRM were defined in the same way. Estimates of fixed-effect parameters and their 95%
186 confidence intervals under the full and optimal NLMM are shown in Tables 1(a) and (b),
187 respectively; those under the full and optimal NLRM are shown in Tables 2(a) and (b), respectively.

188 **Table 1. Estimated fixed effects parameters in the full NLMM (a) and those in the optimal**
189 **NLMM (b).**

190 (a)

Full NLMM						
Parameter	Estimate	SE	95% Confidence Interval		p-value	
			Lower bound	Upper bound		
β_1	0.069	0.015	0.041	0.098	0.000	**
β_2	-0.007	0.003	-0.012	-0.001	0.023	*
ω_1	10.391	4.808	0.968	19.815	0.015	*
ω_2	0.082	0.166	-0.243	0.407	0.310	
ξ_0	4.326	0.020	4.288	4.364	0.000	**
ξ_1	0.003	0.041	-0.076	0.083	0.468	
ξ_2	-0.008	0.022	-0.052	0.035	0.353	

191

** : $p < 0.01$, * : $0.01 \leq p < 0.05$

192

Estimated random effect parameters: $(\psi^2, \phi^2, \sigma^2) = (0.0018, 0.0019, 0.0015)$

193

Log-likelihood: 643.47, AIC: -1266.94, BIC: -1227.44

194

195

196 (b)

Optimal NLMM						
Parameter	Estimate	SE	95% Confidence Interval		p-value	
			Lower bound	Upper bound		
β_1	0.066	0.016	0.033	0.098	0.000	**
β_2	-0.006	0.003	-0.012	0.001	0.036	*
ω_1	9.063	2.949	3.283	14.843	0.001	**
ξ_0	4.319	0.014	4.290	4.347	0.000	**

197

** : $p < 0.01$, * : $0.01 \leq p < 0.05$

198

Estimated random effect parameters: $(\psi^2, \phi^2, \sigma^2) = (0.0018, 0.0019, 0.0015)$

199

Log-likelihood: 642.90, AIC: -1271.80, BIC: -1244.15

200

201 **Table 2. Estimated fixed effect parameters in the full NLRM (a) and those in the optimal**
 202 **NLRM (b).**

203 (a)

12

Full NLRM						
Parameter	Estimate	SE	95% Confidence Interval		p-value	
			Lower bound	Upper bound		
β_1	0.075	0.023	0.030	0.120	0.001	**
β_2	-0.006	0.005	-0.016	0.004	0.104	
ω_1	3.922	1.404	1.170	6.674	0.003	**
ω_2	0.574	0.447	-0.303	1.450	0.100	
ξ_0	4.333	0.007	4.320	4.346	0.000	**
ξ_1	0.003	0.016	-0.028	0.033	0.435	
ξ_2	-0.011	0.008	-0.027	0.006	0.107	

204

** : $p < 0.01$, * : $0.01 \leq p < 0.05$

205

Estimated residual variance: $\hat{\sigma}^2 = 0.00502$

206

Log-likelihood: 744.091, AIC: -928.17, BIC: -883.56

207

208

209

210

(b)

Optimal NLRM						
Parameter	Estimate	SE	95% Confidence Interval		p-value	
			Lower bound	Upper bound		
β_1	0.049	0.005	0.039	0.059	0.000	**
ω_1	5.973	1.726	2.590	9.356	0.000	**
ξ_0	4.334	0.006	4.323	4.345	0.002	**
ξ_2	-0.010	0.003	-0.016	-0.004	0.000	**

211

** : $p < 0.01$, * : $0.01 \leq p < 0.05$

212

Estimated residual variance: $\hat{\sigma}^2 = 0.0058$

213

Log-likelihood: 742.12, AIC: -930.25, BIC: -899.02

214

215

3.1.2. Estimation of the random effects parameters. In the optimal NLMM,

216

variances of the random effects due to individual differences, daily variation, and measurement

217 error were 0.0018, 0.0019, and 0.0015, which account for 35%, 36%, and 29% of the total variance,
218 respectively. Predictions of individual differences $(\hat{\delta}_1, \hat{\delta}_2, \dots, \hat{\delta}_{16})$ and those of daily fluctuation
219 $(\hat{\eta}_{-3}, \hat{\eta}_{-2}, \dots, \hat{\eta}_{20})$ were obtained by calculating posterior means. The predictions $\hat{\delta}_i$ in each of the
220 four groups (control group and three irradiated groups) and the predictions $\hat{\eta}_t$ by day are shown in
221 panels (a) and (b) of Fig 3, respectively.

222

223 **Fig 3. Predictions of random values.** Predictions of random values by individual $\hat{\delta}_i$ by group are
224 shown in panel (a) and predictions of random values by day $\hat{\eta}_t$ are shown in panel (b).

225

226 Residuals in the optimal NLMM and in the optimal NLRM are given by $y_{it} - \hat{f}(t | D_i, \theta) - \hat{\delta}_i - \hat{\eta}_t$ and
227 $y_{it} - \hat{f}(t | D_i, \theta)$, respectively. The standard deviations of residual errors in the optimal NLMM
228 and optimal NLRM were 0.038 and 0.071, respectively. The distributions of residuals in the NLMM
229 and NLRM are shown in Fig 4.

230

231 **Fig 4. Parallel boxplots of residual errors in the non-linear mixed model (NLMM) and ordinary**
232 **non-linear regression model (NLRM).**

233

234 **3.2. Comparison of goodness of fit of the NLMM and the NLRM**

235 There is a large difference between the AICs of the optimal NLMM and the optimal NLRM,
236 which were -1271.80 and -930.25 , respectively (See Table 1 (b) and Table 2 (b)). The measurement
237 error variances of the NLMM and NLRM were 0.0015 and 0.0058 (See Table 1 (b) and Table 2 (b)).
238 Therefore the fit of the NLMM was preferable to that of the NLRM in terms of prediction and accuracy.
239 The estimated time dependency of activity in each group under the optimal NLMM is shown in Fig 5.

240

241 **Fig 5. Estimated mean trends of daily locomotor activity in rats by dose group under the**
242 **optimal NLMM..**

243

244 In each of the irradiated groups, activity decreased immediately after irradiation but recovered to the
245 pre-irradiation level within a few days with a common recovery rate irrespective of dose.

246 **Discussion**

247 One of the advantages of using the more complex NLMM structure, as demonstrated in this
248 paper, is that a second-order dose dependency could be detected in the initial decrease, which was
249 not found with the NLRM (which estimated a linear dependency). Estimated magnitudes of initial
250 decreases at $t = 0$ by dose group and their 95% confidence intervals in the optimal NLMM and
251 those in the optimal NLRM are shown in Fig 6.

252

253 **Fig 6. Fitted dose response curves from the optimal NLMM and the optimal NLRM.** The
254 estimated magnitudes of decrease at $t = 0$ by dose group and their 95% confidence intervals and
255 fitted dose-response curves with dotted line from the NLMM and the NLRM are shown in panels (a)
256 and (b), respectively. Cross marks show observed data of individual rats. The fitted dose-response
257 curve from the optimal NLMM was a downward convex quadratic curve.

258

259 The plots of predictions of individual differences $\hat{\delta}_i$ by dose group (Fig 3 (a)) show that the
260 assumption of homoscedasticity for distributions of individual difference between the four dose
261 groups seems to be satisfied. This means that the random assignment of rats to the four groups was
262 effective in terms of individual differences. The plots of predictions of time-dependent daily
263 fluctuation $\hat{\eta}_t$ (Fig 3 (b)) show that the assumption of independency of each of the random variables
264 η_t seems to be satisfied. The Durbin Watson statistic [26] for $\hat{\eta}_t$ was 2.33 (p-value 0.902),
265 indicating that no strong autocorrelation is observed in daily fluctuation.

266 Because acute changes were the focus in this experiment, longer observation was not
267 performed, but it is necessary to investigate late effects. The irradiation was a single and sub-lethal
268 dose, so it is considered that damage was acute, disappearing in a short period of time, and
269 resilience to allow recovery from the damage was not affected by irradiation. The effects of chronic
270 low dose exposure remain as future issues to be addressed. As one important example of the need

271 for assessing effects of chronic exposure, a giant earthquake of magnitude M9 struck East Japan on
272 March 11, 2011. Subsequently a ‘tsunami’ engulfed the Fukushima Daiichi Nuclear Power Plant
273 (FDNPP). As a result, FDNPP reactors 1-3 suffered meltdown and significant amounts of radioactive
274 materials have been released into the environment [27]. The dose to the public is estimated to be low
275 [28], but many Japanese people are worried about the resulting health effects of chronic low dose
276 exposure.

277 In the present study, effects of irradiation on behavior of rats were investigated efficiently,
278 despite a small number of animals with large individual differences. This was achieved by using a
279 statistical method that accounts for inter-animal differences and daily fluctuation in activity—a
280 non-linear mixed model fit to repeated measurements. With such an efficient approach, we were able
281 to demonstrate a temporary, but dose-dependent, decrease in activity following irradiation and a
282 dose-independent common recovery rate. The statistical framework for analyzing longitudinal
283 locomotor data in this study should be generally applicable to other repeated measurement data with
284 similar structure.

285 **Supporting Information**

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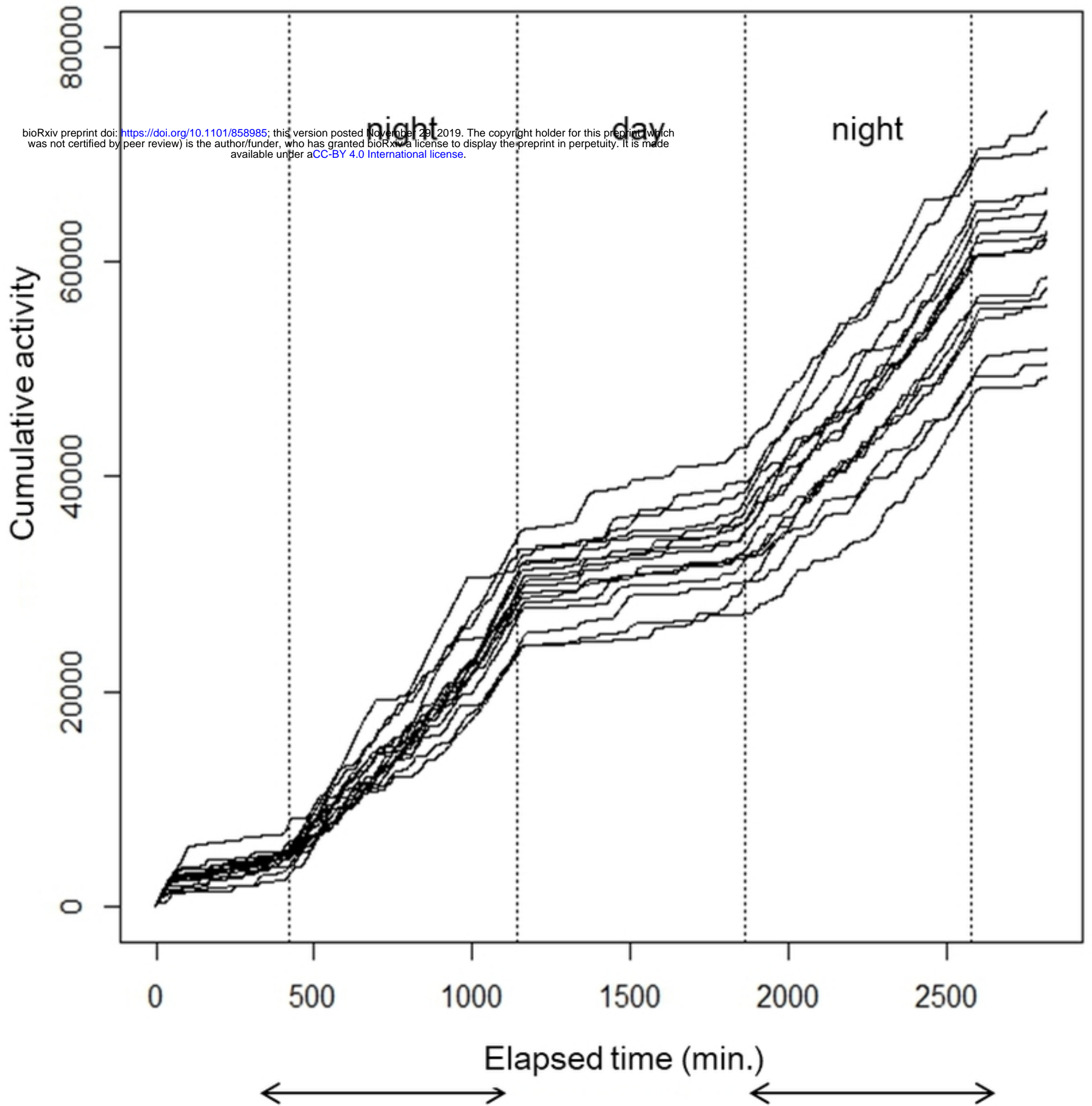
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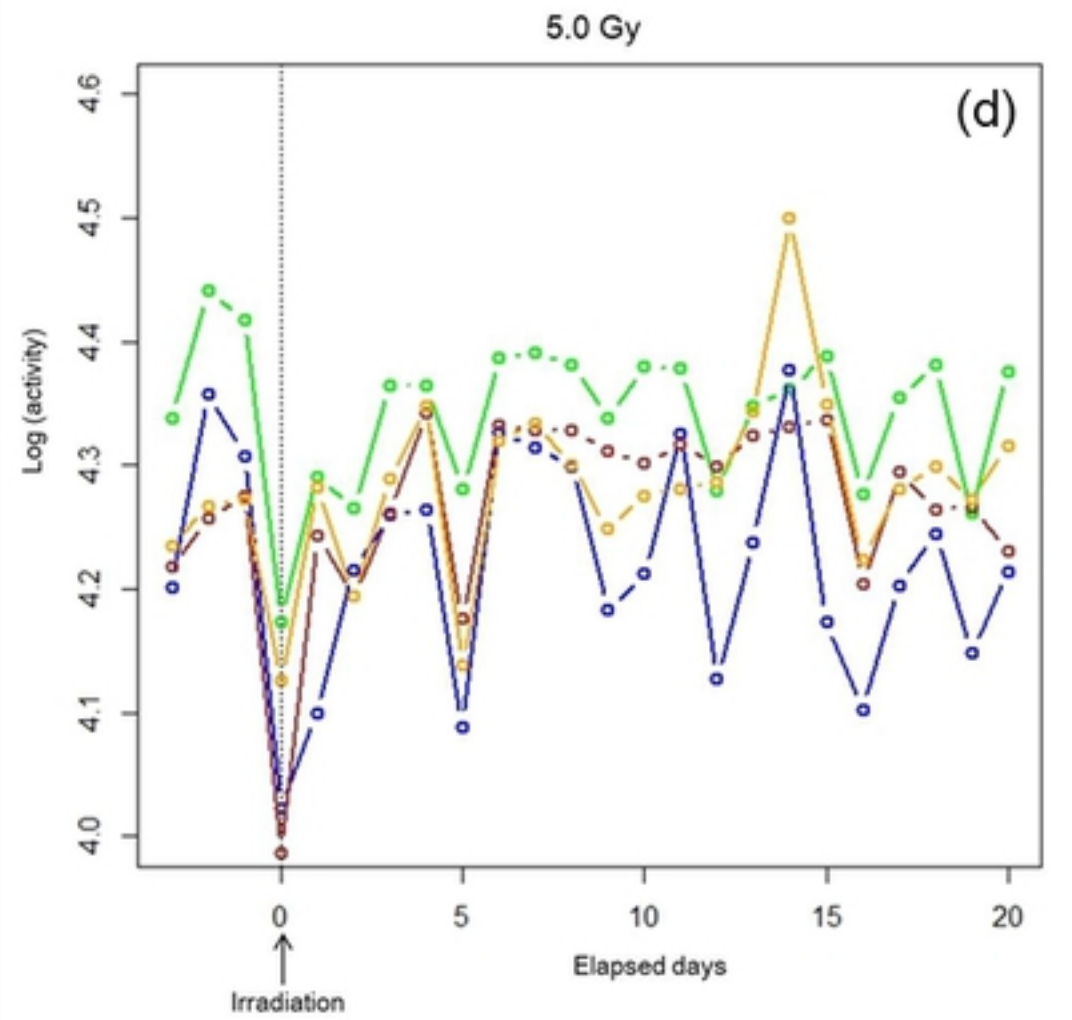
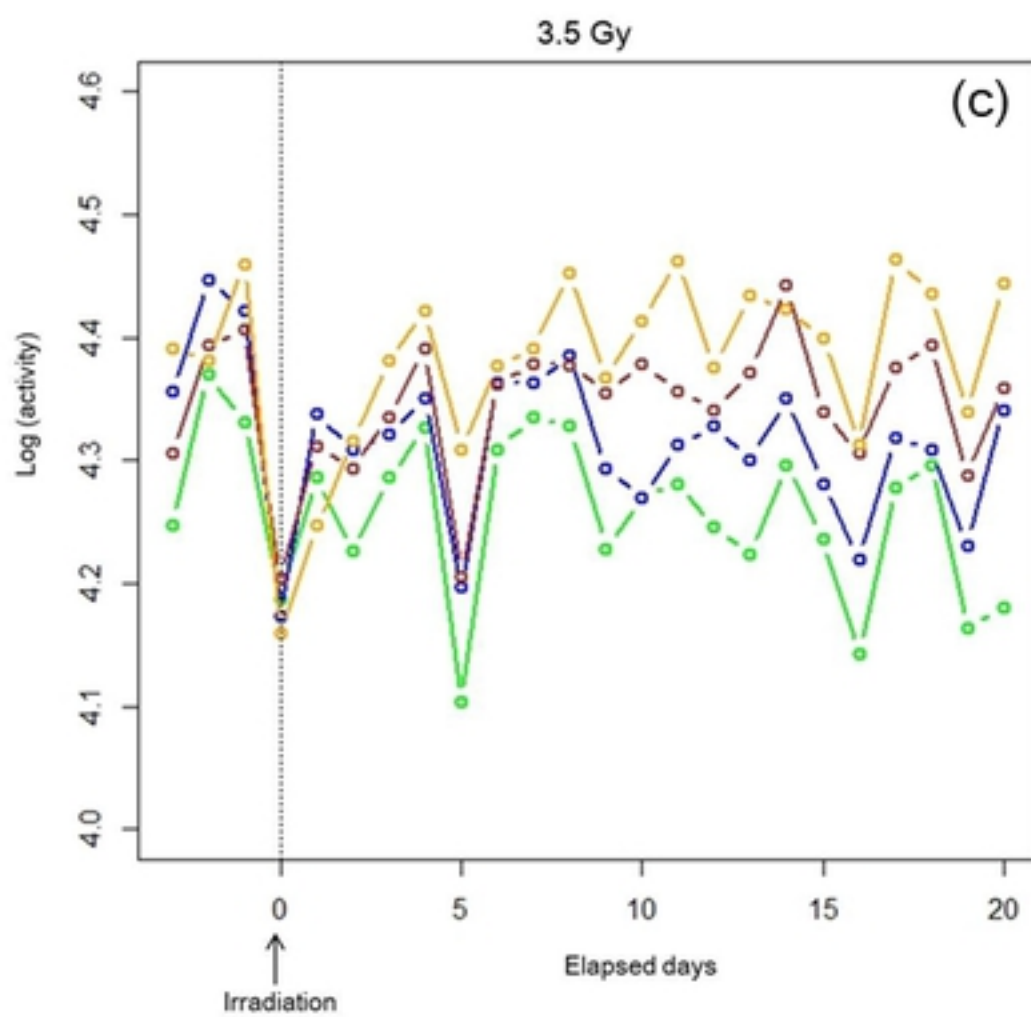
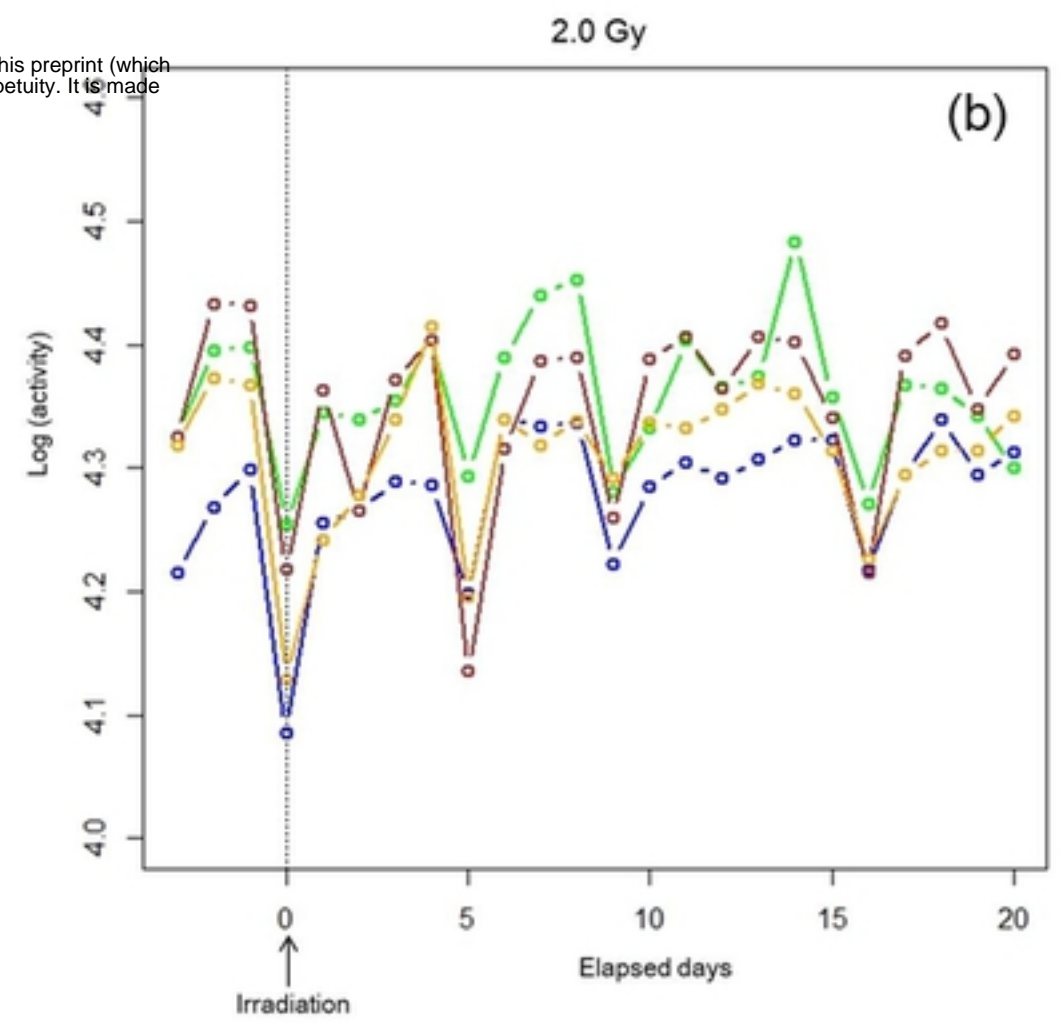
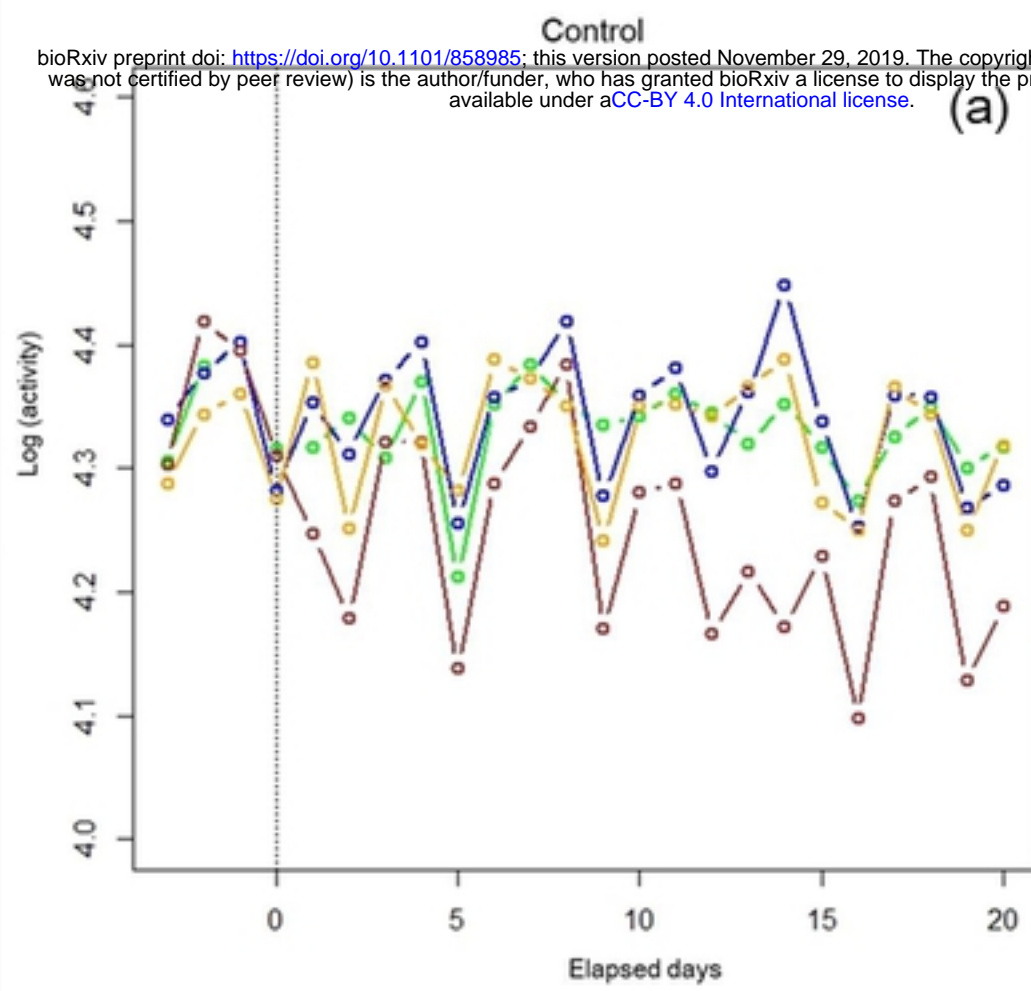
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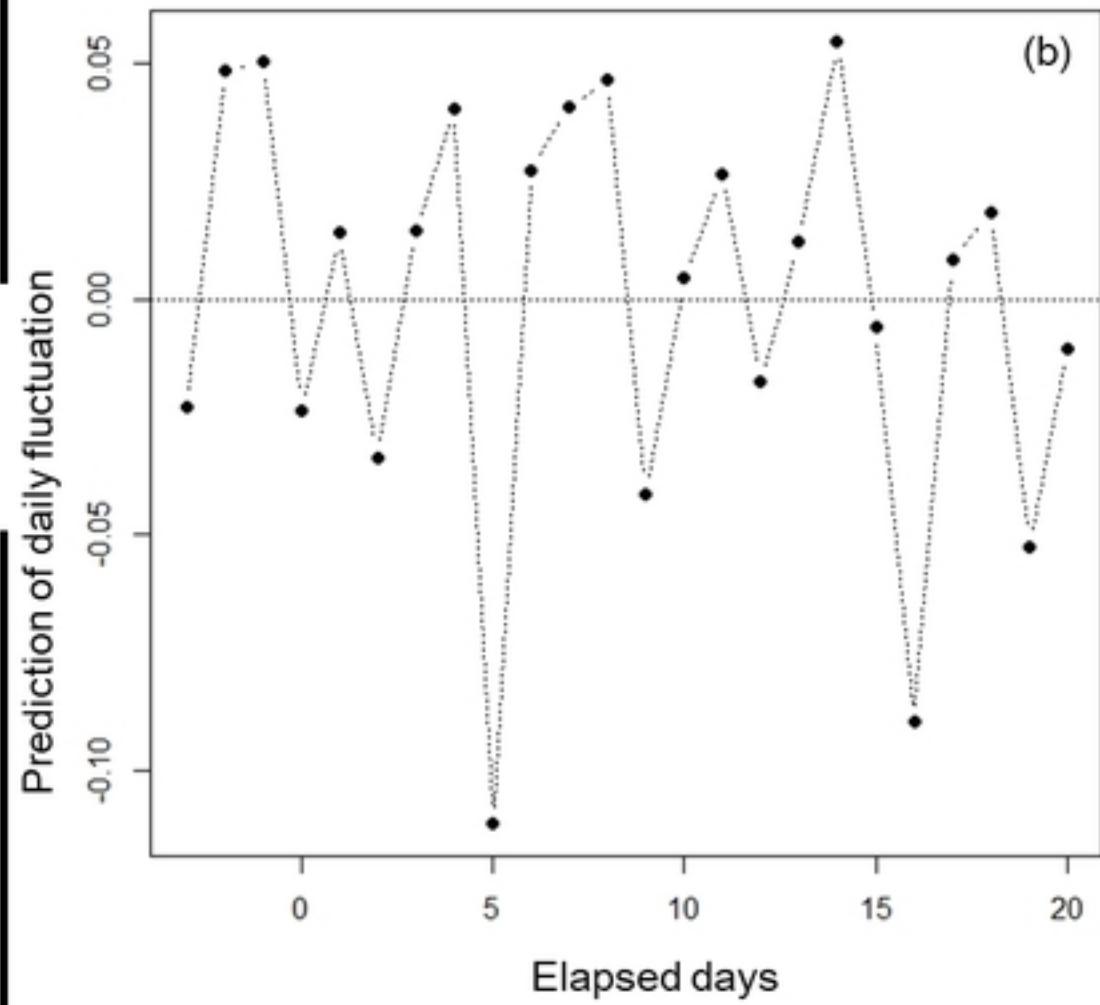
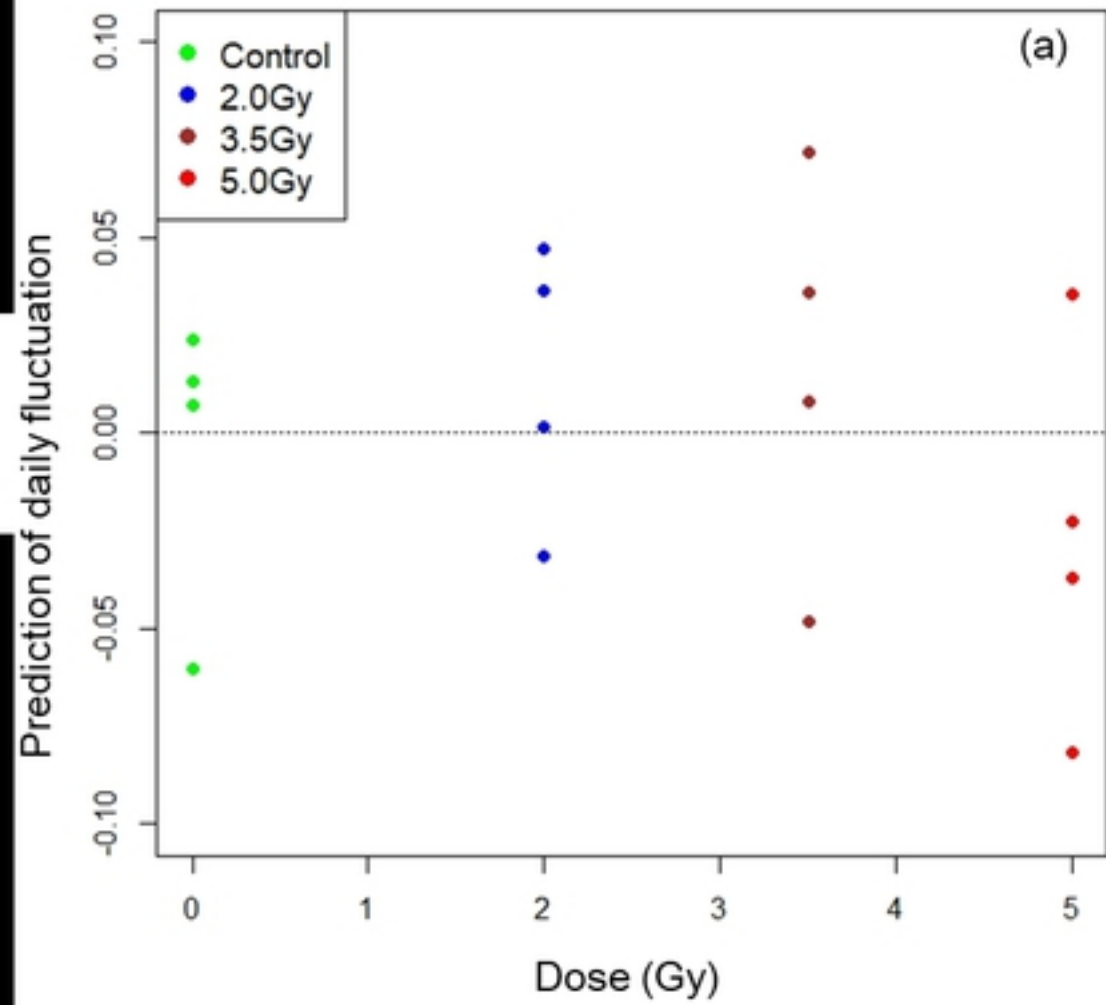
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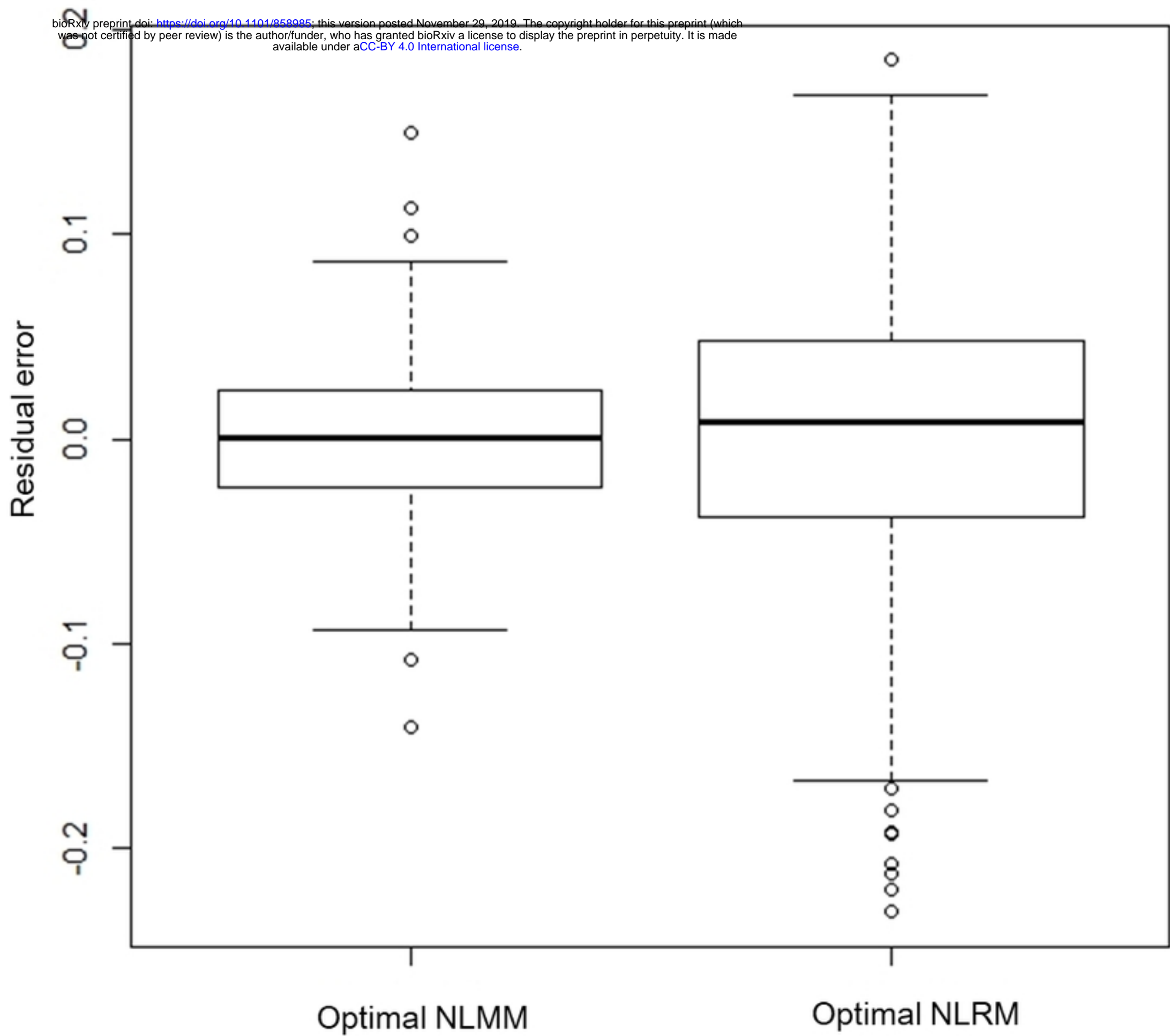
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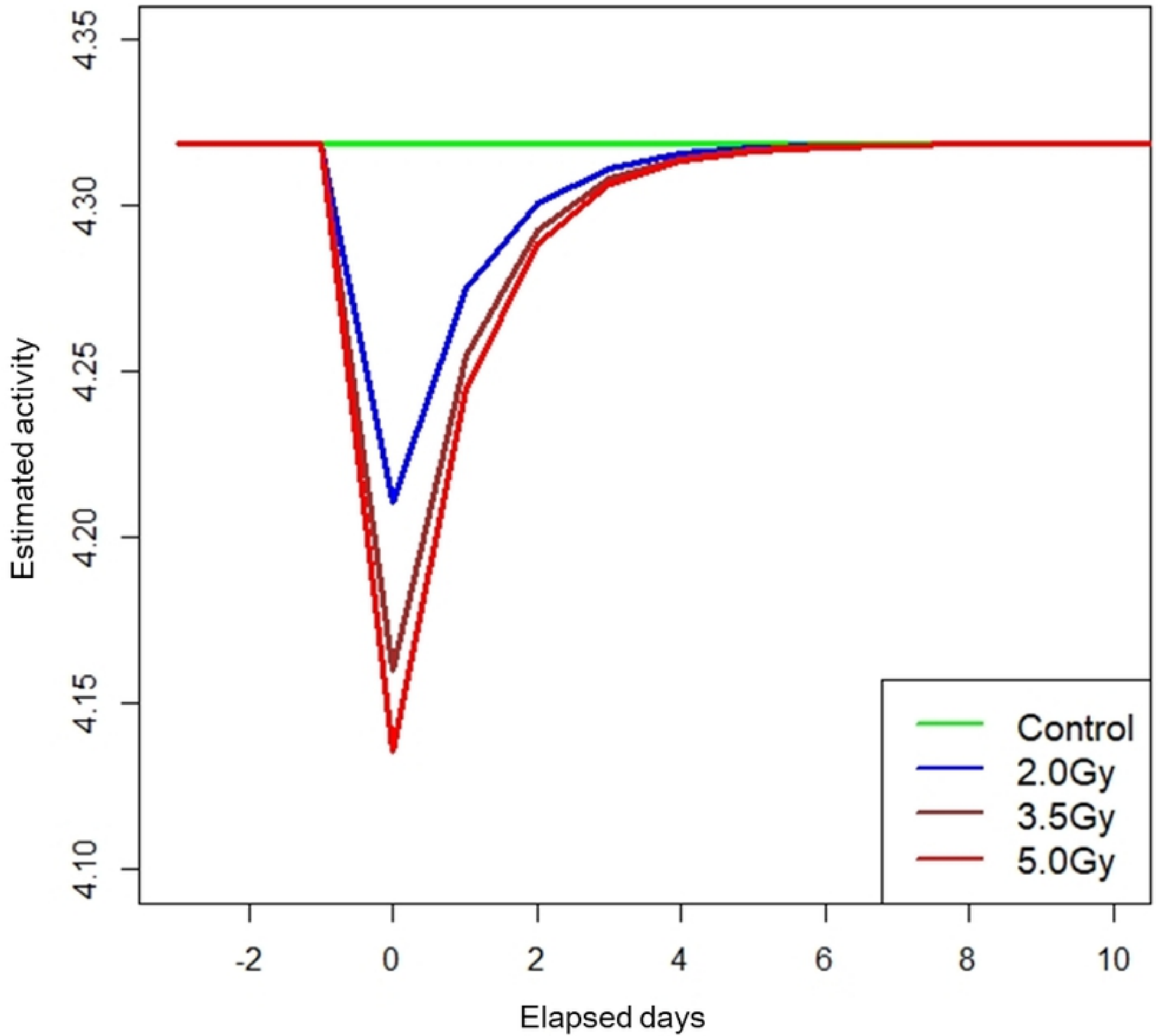
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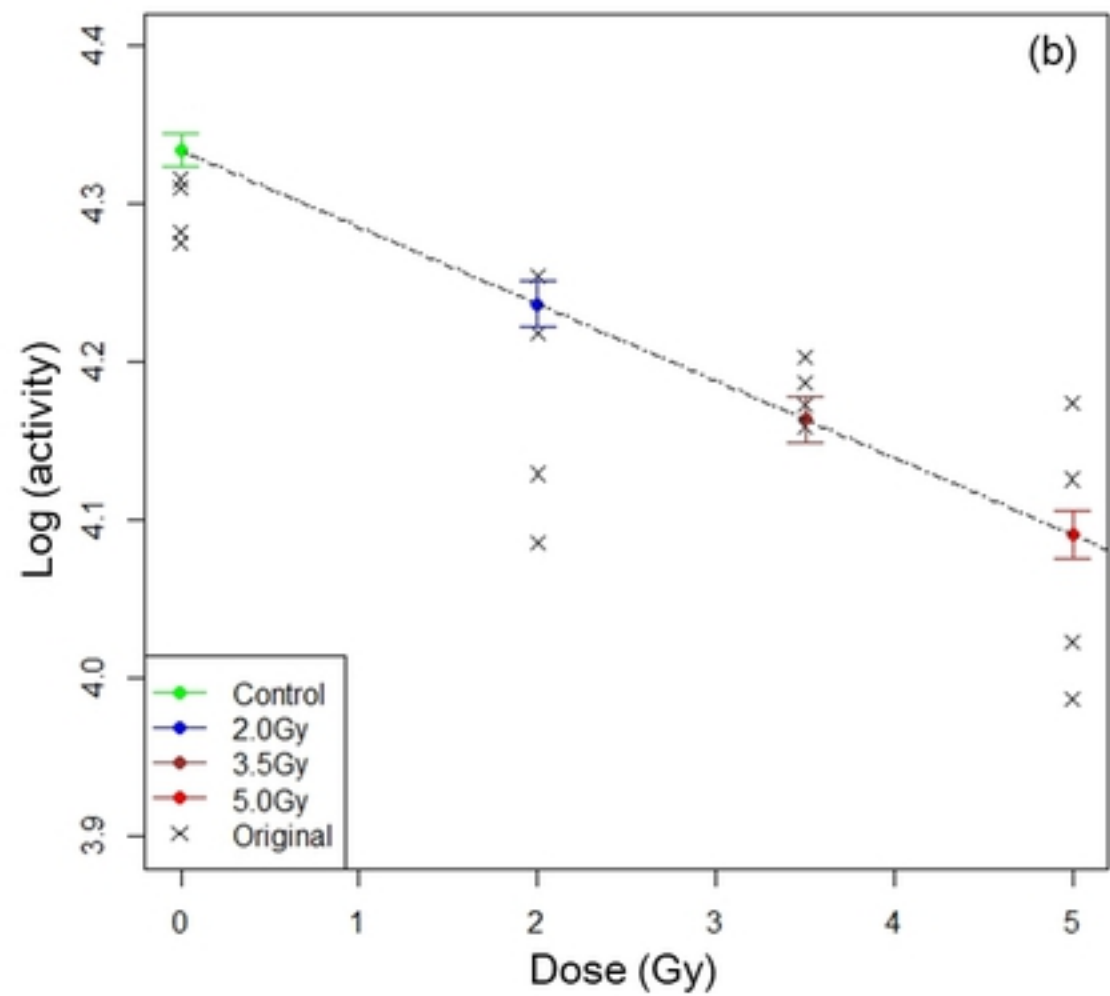
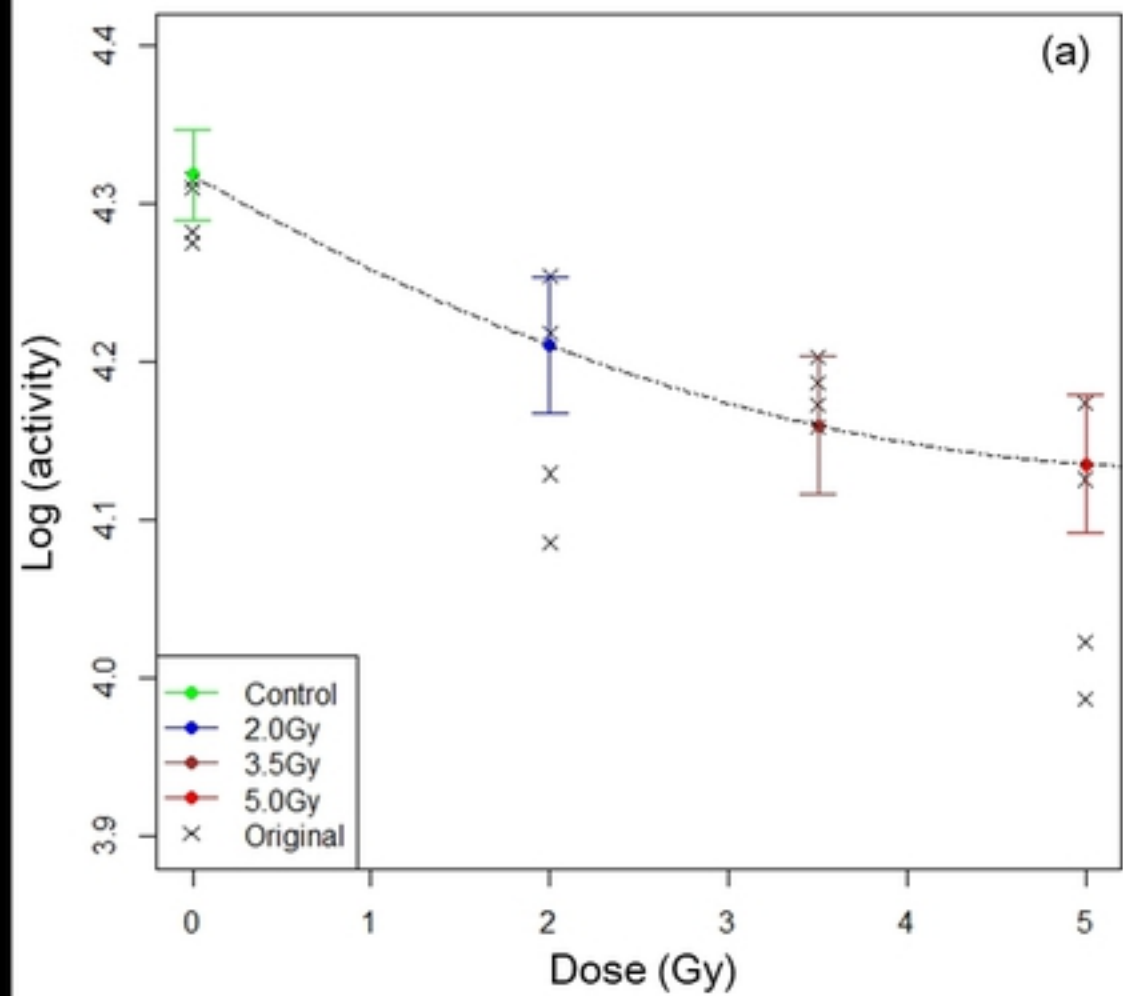
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