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# Radioactive Contamination Of Natural Biological Systems: Oxidative Balance And Genetic Stability

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RADIOACTIVE CONTAMINATION OF NATURAL BIOLOGICAL SYSTEMS:  
OXIDATIVE BALANCE AND GENETIC STABILITY

by

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## DEDICATION

To my grandparents, Dr. Galina N. Dobrovol'skaya and Dr. Sc. Leonard O. Einor,  
and to my great-grandparents, Dr. Sc. Zoya S. Rumyantseva and Dr. Sc. Olgerd L. Einor.

But are those genes, really?

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## ABSTRACT

The global demand for nuclear energy grows, and so do the risks of an accidental release of nuclear products into the environment. Therefore, understanding the health and ecological effects of contamination by radionuclides should be of great interest for biomedical specialists and the public. Empirical studies unequivocally show that high doses of ionizing radiation (IR) shift the redox-balance of many biological systems and increase the rates of genetic errors. However, the effects of chronic low doses of radiation vary significantly between tissues and between species. The present dissertation comprises the results of experimental and meta-analytical research of a variety of biological effects of environmental contamination by radioactive isotopes.

In the review part (Chapter 1), I performed a rigorous literature analysis of the environmental consequences of the three most serious nuclear accidents – the Fukushima Daiichi nuclear power plant (NPP) accident in Japan (2011); meltdown at the Chernobyl NPP in 1986; and the explosion at the Mayak plutonium production site (both former USSR) in 1957. The results of the meta-analysis demonstrate significant effects of the low (<0.1 Sv) doses of IR exposure on the redox systems of humans as well as animals from the surrounding environment affected by the nuclear accidents. A significant heterogeneity in radiation effects was observed between species and tissues, suggesting different sensitivity of biological models to IR.

In the experimental part of this thesis (Chapter 2&3), I presented the results of several tests, by which I aimed to detect the detrimental genetic effects of low dose IR. I tested for the presence of radiation-induced damage in DNA and related that to radionuclide body burdens and fitness of a model animal. The bank vole was used as a vertebrate model and the wild downy dragonfly as an invertebrate model. Both species were sampled in the Chernobyl Exclusion Zone (CEZ), as it remains significantly contaminated by the biologically effective isotopes: cesium-137 and strontium-90. In the results, I demonstrate that natural variation in comet-visualized DNA damage exceeds the additive effect of damage from ionizing radiation. It was also shown that accounting for morphological co-variates, such as sex and age, and random co-variates, such as sampling period, is important in prediction of genetic damage using the comet assay.

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## LIST OF SYMBOLS

$df$	Degrees of freedom
$g$	Hedge's estimate of effect size
$d$	Cohen's estimate of effect size
$\mu$	Population mean
$m$	Sample mean
$M$	Sample median
$\sigma$	Population variance
SD	Standard deviation of a sample mean

## LIST OF ABBREVIATIONS

AB	Alkaline Buffer
AIC	Akaike Information Criterion
AO	Antioxidants
AOS	Antioxidant System
CA	Comet Assay
CAT	Catalase
CEZ	Chernobyl Exclusion Zone
CN	Chernobyl – Northern Area
CSE	Chernobyl – South Eastern Area
CSW	Chernobyl – South Western Area
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetate
ES	Effect Size
FA	Fluctuating Asymmetry
GLMM	Generalized Linear Mixed Model
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GST	Glutathione-S-Transferase
GSSG	Glutathione Disulfide

IR.....	Ionizing Radiation
LB .....	Lysis Buffer
LDIR .....	Low Doses of Ionizing Radiation
MDA .....	Malondialdehyde
NB.....	Neutralizing Buffer
PK .....	Proteinase K
ROS.....	Reactive Oxygen Species
SOD.....	Superoxide Dismutase
WBC .....	White Blood Cells

## CHAPTER 1

### IONIZING RADIATION, OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE:

#### A META-ANALYSIS<sup>1</sup>

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<sup>1</sup> Einor, D., Bonisoli-Alquati, A., Costantini, D., Mousseau, T., & Møller, A. (2016). *Science of the Total Environment*, 548, 463-71.



## ABSTRACT

One mechanism proposed as a link between exposure to ionizing radiation and detrimental effects on organisms is oxidative damage. To test this hypothesis, we surveyed the scientific literature on the effects of chronic low-dose ionizing radiation (LDIR) on antioxidant responses and oxidative damage. We found 40 publications and 212 effect sizes for antioxidant responses and 288 effect sizes for effects of oxidative damage. We performed a meta-analysis of signed and unsigned effect sizes. We reported large unsigned effects for both categories (0.918 for oxidative damage; 0.973 for antioxidant response). Mean signed effect size weighted by sample size was 0.276 for oxidative damage and -0.350 for antioxidant defenses with significant heterogeneity among effects for both categories, implying that ionizing radiation caused small to intermediate increases in oxidative damage and small to intermediate decreases in antioxidant defenses. Our estimates are robust as shown by very high fail-safe numbers. Species, biological matrix (tissue, blood, sperm) and age predicted the magnitude of effects for oxidative damage as well as antioxidant response. Meta-regression models showed that effect sizes for oxidative damage varied among species and age classes, while effect sizes for antioxidant responses varied among species and biological matrices. Our results are consistent with the description of mechanisms underlying pathological effects of chronic exposure to LDIR. Our results also highlight the importance of resistance to oxidative stress as one mechanism that leads to species to flourish or suffer in LDIR-contaminated areas.

## INTRODUCTION

Low-dose ionizing radiation (LDIR) is the type of low-rate chronic irradiation that does not induce adverse toxic effects (National Council on Radiation Protection, NCRP, 1987). However, exposure to LDIR can accelerate cellular senescence via increasing activity of reactive oxygen species (ROS) and disruption of biopolymers (Loseva et al., 2014). Because the power of exposure decreases with the distance to the source, a bigger potential hazard comes from radiological agents being ingested or inhaled.

Industrial and military use of radioactive materials inevitably released these in ecosystems. Early monitoring studies of humans and biota in the ecological aftermaths of usage of nuclear weapons as well as radiation-related accidents (e.g. in Kyshtym, Russian Federation), suggested elevated health risks and mortality rates in humans and some mammals associated with high acquired doses after chronic exposure to LDIR (Sakata et al., 2012; Lushnikova et al., 1997; Mozolin et al., 2008; Shoikhet et al., 1999; Grigorkina and Olenev, 2013; Grigorkina and Pashnina, 2007). These studies also suggested that the effects of direct exposure to ionizing radiation were exacerbated by incorporation of soluble radioactive elements (Ivannikov et al., 2002). Recent ecological studies of the Chernobyl and Fukushima catastrophes confirmed this and demonstrated high variability in such effects among taxa.

Along with a high frequency of morphological abnormalities (Akimoto, 2014; Hiyama et al., 2012, 2013; Møller et al., 2007) and tumors (Møller et al., 2013), and an overall decline in population abundance (Møller and Mousseau, 2007, 2009; Møller et al., 2012), these effects included high rates of genetic aberrations in somatic (AlAmri et al., 2012; Bonisoli-Alquati et al., 2010; Møller et al., 2013) and germline cells (Ellegren et al.,

1997). Moreover, variation exists across species in their biochemical and genetic responses to increasing background radiation (Galván et al., 2014; Hinton et al., 2007).

Overall, attempts to rigorously monitor human populations in Ukraine, Belarus and Russia following the Chernobyl accident have been scattered at best (Edwards et al., 2004; Yablokov et al., 2009). Nonetheless, these studies showed that workers involved in the cleanup operations (the so-called ‘liquidators’), who were exposed to much higher doses than evacuated civilians, demonstrated elevated frequencies of genetic abnormalities (Moysich et al., 2002; Sevan’kaev et al., 2005), solid cancers and cardio-vascular diseases (Cardis and Hatch, 2011; Serdiuk et al., 2011). An elevated fraction of evacuated adolescents and young adults suffered from thyroid cancer (Demidchik et al., 2007). At the same time, epidemiological studies with small cohorts and small and non-representative control groups carried out years after the catastrophe did not yield sufficient evidence to support the hypothesis of radiation-associated mortality linked to the Chernobyl accident (Serdiuk et al., 2011; Weinberg et al., 2001).

These findings emphasize the interest of studying variation in health effects in the context of chronic LDIR, as well as in-depth analyses of compromises between radiation effects and cellular radiation-defense mechanisms. Results of such studies are important and may be used in radiation protection and for defining safety requirements, particularly given the current debates about the shape of dose-response curves describing radiation-related effects and the severity of radiation injury (Ryan, 2012).

When absorbed by living cells, ionizing radiation can induce direct breakage in the chemical bonds of biological macromolecules. Ionizing radiation can also affect proteins, nucleic acids and complex lipids as a result of the generation of reactive oxygen species

(ROS) via radiolysis of water or alteration of mitochondrial functions (Kam and Banati, 2014). ROS are a diverse group of chemical species, which naturally occur in cells, where they perform important signaling functions (Azzam et al., 2011; Murphy et al., 2011). ROS activity is controlled by a number of enzymatic and non-enzymatic antioxidants. The inability to balance the increased generation of ROS by antioxidant mechanisms results in oxidative stress, a complex stressor for cells that manifests as increased oxidative molecular damage to biomolecules, e.g. oxidation of lipids, oxidative modification of nitrogenous bases etc. (Halliwell and Gutteridge, 2007; Jones, 2006). In turn, oxidative damage may promote the emergence of pathological states, accelerated cell aging and apoptosis (Halliwell and Gutteridge, 2007; Spitz et al., 2004). In numerous invertebrate and vertebrate species, oxidative damage may result in reduced growth, fertility and survival (Costantini, 2014).

The association between LDIR and the generation of reactive species has been widely described (Azzam et al., 2011; Smith et al., 2012). The role of ionizing radiation in generation of ROS is well explained as the correlation between genetic damage and oxidative damage (e.g. Costantini, 2014; Galván et al., 2014). Oxidative damage might be one mechanism underlying several of the detrimental effects of radiation. Thus, we aim to test this hypothesis using meta-analysis. The root of the controversy relates to the manifestation of a given symptom or ailment as a consequence of the three-way interaction of increased concentrations of ROS, decreased activity of antioxidant enzymes, and genetic damage associated with increased background radiation (Spitz et al., 2004), especially when disease is followed by another medical condition, like malnutrition, inflammatory disease or respiratory malfunction. However, it is important to note that radionuclides do

not only generate damage through radiation, but also through their catalytic activity (the Fenton reaction) (Halliwell and Gutteridge, 2007). In addition, while several studies have documented increased oxidative damage and reduced antioxidant defenses in humans and wild populations of animals chronically exposed to LDIR (e.g. Bonisoli-Alquati et al., 2010), other studies have shown the potential for animals to adapt their antioxidant system to chronic exposure to LDIR (Galván et al., 2014). In addition, theoretical calculations and lack of accurate dosimetry have called into question findings of increased oxidative stress from exposure to LDIR (Smith et al., 2012).

Here we assess the effects of chronic exposure to LDIR from radioactive contamination. We aim at exploring the insights of long-term metabolic processes, such as antioxidant function and oxidative damage, of individuals affected by chronic irradiation caused by radioactive contaminants. We collected exhaustive data from radiobiological studies in the Russian and the English language scientific literature, and combined published evidence into a meta-analysis of the effects of chronic radiation exposure on markers of oxidative damage and antioxidant protection. Our aim was to test whether high environmental radioactivity would lead to higher oxidative damage and lower antioxidant defenses in exposed organisms. Meta-analysis is a powerful tool for quantitatively summarizing such research, especially when there is apparent heterogeneity in research findings (Arnqvist and Wooster, 1995; Hedges and Olkin, 1985; Koricheva et al., 2013).

We expected factors related to study design, age and model organism to explain variation across studies and species in the relationship between LDIR exposure and oxidative damage. Hence, we also tested whether biological matrices and species differed in their response to radiation. Different organs and tissues can be differentially exposed

and/or sensitive to radiation exposure, depending on the metabolic fate of radionuclides. Juveniles and adults can also differ in their sensitivity, with individuals at early developmental stages generally being more sensitive to increased radiation because of their immature antioxidant system (Costantini, 2014; Lu&Finkel, 2008) and due to potential hazardness of the damage being accumulated in their stem cell progeny (Liu et al., 2014). Finally, variation across species in exposure and sensitivity to LDIR would allow us to identify species that can serve as sensitive bio-indicators of the effects of LDIR, and model how such effects translate into potential risk for humans and other species (Møller and Mousseau, 2015).

## MATERIALS AND METHODS

### *Literature search and data sets*

We made an exhaustive literature search of both correlative and experimental studies on Web of Science relying on the following keywords: "radioactive contamination", "increased background radiation", "occupation exposure", "Chernobyl" in combination with "oxidative stress", "reactive oxidative species", "lipid peroxidation", "peroxide radicals" and "antioxidant". Once having located these papers we tested if they fulfilled our inclusion criteria. We also searched the reference lists of all identified publications in an attempt to locate additional publications. Because many nuclear accidents have occurred in the former Soviet Union, a large number of publications have appeared in Russian, Belorussian and Ukrainian. Although such publications are often neglected or deliberately omitted from meta-analyses, we made a concerted effort to identify such publications.

We included (1) ecological or biomedical surveys of individuals exposed to high environmental radiation levels; (2) studies examining the relationship between acquired dose (external; ingested etc.) and markers of ROS metabolism; (3) studies reporting at least one statistical test, which compared non-exposed and exposed individuals; and (4) studies from which those data could be extracted and converted into effect sizes. Exclusion criteria were (1) studies that involved radiation therapy (e.g. for oncology treatment); (2) studies that concerned treatment of radiation-affected people; and (3) studies that involved short-term exposure to toxic doses of ionizing radiation. We present a PRISMA diagram showing the number of initial publications and the subsequent publications excluded from the final sample of 40 studies (Fig. 1.1). This resulted in 212 effect sizes for antioxidant response and 288 effect sizes for oxidative damage. All studies found by March 31 2015 were included in the analyses, and all supplementary data is published online in a research network repository at ResearchGate website. Copies of most of these papers, some of which are difficult to obtain electronically, have been posted on a website (<http://cricket.biol.sc.edu/oxi-stress>).

### *Method of Meta-analyses*

A meta-analysis was performed using signed and unsigned effect sizes to estimate a direction and magnitude of LDIR effects on oxidative status. The data were analyzed using the software Meta-Win (Rosenberg et al., 2000). We estimated effect sizes in terms of Hedges  $g$  by using standard procedures. For data that contained estimated means and standard deviations we used the formula by Hedges and Olkin, shown in Equation 1.1 (1985):

$$g = \frac{M_{\text{experiment}} - M_{\text{control}}}{SD_{\text{pooled}}^*}$$

**Equation 1.1**

where Hedges pooled standard deviation was estimated as in Equation 1.2:

$$SD_{\text{pooled}}^* = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2}{n_1 + n_2 - 2}}$$

**Equation 1.2**

where sample standard deviations were  $SD_1$  and  $SD_2$  and sample sizes were  $n_1$  and  $n_2$  respectively for the two samples. If a standard error was reported, it was simply transformed into SD as follows in the Equation 1.3:

$$SD = \frac{SE}{\sqrt{n}}$$

**Equation 1.3**

For transformation of F-values and Pearson's correlation coefficient  $r$  we estimated effect sizes in terms of Cohen's  $d$  using equations in Rosenthal (Rosenthal, 1994). In particular, we converted  $F$ -values from a one-way ANOVA (or the main effect of a two- or more way ANOVA; here ionizing radiation) into Cohen's  $d$  (Equation 1.4):

$$d = \sqrt{F \left( \frac{n_i + n_c}{n_i n_c} \right) \left( \frac{n_i + n_c}{n_i + n_c - 2} \right)}$$

**Equation 1.4**

Pearson's  $r$  was converted into Cohen's  $d$  as in Equation 1.5:



$$d = \frac{2r}{\sqrt{1 - r^2}}$$

**Equation 1.5**

Likewise, the variance of Cohen's  $d$  was defined as follows in Equation 1.6:

$$\sigma_d^2 = \left( \frac{n_i + n_c}{n_i n_c} + \frac{d^2}{2(n_i + n_c - 2)} \right) \left( \frac{n_i + n_c}{n_i + n_c - 2} \right)$$

**Equation 1.6**

where  $n_i$  and  $n_c$  were the sample sizes of the two samples. Cohen's  $d$  can be converted into Hedges  $g$  (Equation 1.7) relying on Hedges and Olkin (1985):

$$g \approx d \left( 1 - \frac{3}{4(n_i + n_c) - 9} \right)$$

**Equation 1.7**

Because there was considerable heterogeneity in the number of effect sizes per study, and because of differences in effect size among studies, we used random effect meta-analysis to account for such heterogeneity (Raudenbush, 1994). Variables used in random effect models were subsequently entered in meta-regressions with study as a random effect, and the three fixed effects of species, biological matrix and age as predictors. These models were subsequently reduced by eliminating all predictors that did not explain part of the variance at a level of  $P < 0.10$ . We used  $P < 0.10$  to avoid excluding factors with overall weak effects. Full models including all three predictors all resulted in similar conclusions. All analyses were made using JMP (SAS Institute Inc., 2012).

We interpreted our data as follows: positive ES for an oxidative marker and negative ES for a marker of antioxidant response would indicate a negative impact of LDIR. Likewise, the effect of LDIR would be considered positive if oxidative damage is decreased (negative ES for oxidative marker) and when enzymatic and non-enzymatic antioxidants were increased (positive ES for antioxidant marker), respectively (Costantini and Møller, 2009; Costantini et al., 2011). The analysis of the unsigned effect sizes was performed to determine the magnitude of effect regardless of its direction. Some biomarkers express radiation effects by being up-regulated, while others are down-regulated. Thus, using absolute effect sizes avoids effects on different biomarkers canceling each other out. Effect sizes were considered to be small (Hedges  $g = 0.2$ , explaining 1% of the variance), intermediate ( $g = 0.5$ , explaining 9% of the variance) or large ( $g = 0.8$ , explaining 25% of the variance) as suggested by Cohen (1988).

## RESULTS

### *Markers of response to LDIR: Response in antioxidants and oxidative metabolism*

In the meta-analysis we included enzymatic and non-enzymatic markers of antioxidant response to LDIR. We extracted studies of effects on catalase (CAT,  $n = 55$ ), superoxide dismutase (SOD,  $n = 36$ ), glutathione (GSH,  $n = 17$ ), and glutathione peroxidase (GPX,  $n = 14$ ), as for non-enzymatic markers there were effects on vitamin A (VitA,  $n = 18$ ), vitamin E (VitE,  $n = 18$ ), and carotenoids ( $n = 9$ ). Overall, studies of enzymatic antioxidants accounted for 55% of all markers of antioxidant response.

Markers of oxidative stress included effects on ROS concentration ( $n = 21$ ) and their metabolites – diene and triene MDA conjugates ( $n = 157$ ), thiobarbituric acid reactive

species (TBARS,  $n = 81$ ), and reduced glutathione (GSSG,  $n = 17$ ). Here, metabolic markers accounted for 89% of all markers of oxidative stress.

#### *Variation in effects between relatively high and low doses acquired by humans*

Our analysis included studies on humans that were participating in the cleanup operations after the Chernobyl accident. For some their maximum estimated exposure was at 200 mSv (e.g. Lyashenko. et al., 2000). We compared them to the effects found in studies on other human individuals that were not directly involved in the event, but were also exposed to LDIR. Our analysis suggests none to very small difference between the two groups of studies (antioxidant response:  $F = 0.1$ , d.f. = 1, 124,  $P = 0.752$ ; oxidative damage:  $F = 0.59$ , d.f. = 1, 97,  $P = 0.444$ ).

#### *Summary statistics, mean effect sizes and evidence for publication bias*

Mean effect size for oxidative damage due to radiation and weighted by sample size was 0.277, with a fail-safe number that exceeded 709 (Table 1.1; Fig. F1). The absolute magnitude of oxidative damage was large, and estimated at 0.918. In comparison, mean effect size for antioxidant response weighted by sample size was -0.350 (absolute ES = 0.983) and the fail-safe number was more than 1600 (Table 1.1). In total sample size was 17,332 for 212 effect sizes from studies of antioxidant response and 13,953 for 288 effect sizes from studies of oxidative damage. There was significant heterogeneity for effect sizes for both oxidative damage and antioxidant response (Table 2, Fig. F1).

Effect size was significantly, but weakly positively correlated with sample size for Kendall's rank order correlation for oxidative damage ( $\tau = 0.171, P < 0.0001$ ), but not for antioxidant response ( $\tau = -0.010, P = 0.84$ ). The correlation between effect sizes and their variances was also significant but weak for oxidative damage ( $\tau = -0.149, P = 0.0002$ ), but not for antioxidants ( $\tau = -0.061, P = 0.19$ ). The correlation between effect size and publication year was not significant for oxidative damage ( $r = -0.007, P = 0.87$ ) or for antioxidants ( $r = 0.021, P = 0.67$ ).

### *Moderators of effect size*

We first used Generalized Linear Mixed Models (GLMM) to test for the effects of marker ( $F = 32.67, df = 1, 491.6, P < 0.0001$ , antioxidant:  $-0.393 (0.146)$ ; oxidative damage:  $0.458 (0.155)$ ) and study (variance ratio =  $0.002, 95\% \text{ CI: } 0.026, 0.639, 0.227\%$  of total variance; oxidative damage: variance ratio =  $0.054, 95\% \text{ CI: } -0.126, 0.233, 0.042\%$  of total variance).

Levels of antioxidant response differed significantly among biological matrices (Fig. 3, Table 2). Two out of five matrices had effect sizes (ES) that differed significantly from zero (Table 1.3). While blood had an intermediate negative effect, sperm had a significant intermediate positive effect (Table 1.3). Interestingly, the positive effect for sperm differed significantly from that for eggs, as shown by non-overlapping confidence intervals, and even the sign of mean effect sizes for sperm and eggs was different (Table 1). Biological matrices differed significantly in terms of oxidative damage with significant heterogeneity (Fig. 1.2, Table 1.5). However, significant large absolute effect (i.e.

magnitude) of antioxidant response and oxidative damage was demonstrated for most tissues (Fig. 1.3, Table 1.2).

There was significant interspecific variation in antioxidant response (Fig. 1.5, Table 1.2). Among the 12 species presented here, six showed effect sizes significantly different from zero, with *Homo sapiens*, *Parus major* and *Mus musculus* showing negative effect sizes, while *Myodes rutilus*, *Microtus arvalis* and *Hirundo rustica* showing positive effect sizes (Table 1.2). For oxidative damage three out of seven species (*Apodemus sp.*, *Bos taurus* and *H. sapiens*) showed significant positive intermediate to large effect sizes (Fig. 1.4, Table 1.2). When we used absolute effect sizes, rather than signed ES, we all species had effect sizes significantly different from zero for markers of antioxidant response, while for oxidative damage three species (*Apodemus uralensis*, *Apodemus sylvaticus*, *Myodes glareolus*) did not yield a significant ES.

Age was significantly related to antioxidant response being 35% weaker in juveniles than in adults (Fig. 1.6, Table 1.4). Oxidative damage in juveniles was six-fold larger than in adults (Table 1.2). Thus, juveniles were more susceptible than adults, likely as a consequence of the weaker antioxidant system of juveniles. Interestingly, the magnitude of antioxidant response was significantly higher in adults.

We developed meta-regression models weighted by sample size using study as a random effect. However, none of the random effects accounted for an amount of variance deviating from zero, so instead we used ordinary least squares models. For oxidative damage the model, accounting for 8% of the variance, included significant effects of species and age, but no significant effect of biological matrix (Table 1.5). The effect of

oxidative damage was larger for juveniles than for adults (Table 1.4). For antioxidant response the best-fit model, accounting for 17% of the variance, included significant effects of species, biological matrix and age, with a larger effect in juveniles than in adults (Table 4).

## DISCUSSION

The main findings of this study were significant effects of LDIR on antioxidant status of individuals with a large magnitude of the mean effect (Table 1.1). We found significant heterogeneity in effect sizes among species and biological matrices. There were several-fold stronger effects of oxidative damage in juveniles than in adults. There was little or no indirect evidence to suggest publication bias.

We found mean weighted effect sizes for oxidative damage of 0.237 and for antioxidant response of -0.350. The absolute effect sizes were 0.918 and 0.973 respectively. Cohen (1988) regarded a  $d = 0.50$  to represent an intermediate effect (equivalent to 6% of the variance). Thus the raw effect sizes that we have estimated here can be considered to be small to intermediate, and the magnitude of effects can be interpreted as large. Møller and Jennions (2002) reported when using Hedges  $d$  an average effect size weighted by sample size across all meta-analyses in the biological sciences of 0.721, while the median was 0.595 (in this case the sign of  $d$  was also disregarded).

The majority of our studies were extracted from Russian and Ukrainian sources, which are usually not included in Western bibliographical sources. Thus we were able to test for a difference in effect size between these two categories of effects with the eastern

literature being more likely to be under-represented than the western literature, which is fully indexed in Web of Science as well as in other search engines. However, we found little or no evidence consistent with expectations for publication bias (Møller and Jennions, 2001). Jennions and Møller (2002) reported a general temporal decline in effect size over time. Here we found a weak and non-significant Pearson correlation between effect size and publication year. We also showed a non-significant correlation between absolute effect sizes and publication year. Begg and Mazumdar (1995) proposed a non-parametric correlation between raw effect size and sample as a test of publication bias, and although we showed significant correlations, they differed in sign and were of small magnitude. Funnel plots arise from the reduction in variance in effect size with increasing sample size (Light and Pillemer, 1984). Again, we found little and inconsistent relationships between variance in effect size and sample size (Fig. F1). Thus there was little evidence suggestive of publication bias. Antioxidant responses varied among species, biological matrices and age classes. However, a meta-regression only showed a significant effect of species and biological matrices, when the effects of age classes were excluded as an independent predictor. Blood and eggs had intermediate to large negative effects for oxidative damage and antioxidant responses, while there were weak effects for liver, spleen and brain. The direction of the effect was difficult to evaluate due to high variability in markers studied within biological categories. However, this problem can be alleviated when the sign of the effect size is disregarded in the same model. Such an analysis showed that all biological matrices had significant effects for individuals exposed to LDIR compared to controls (Figs. 1.2-1.4). It was not surprising that brain cells had a large effect. Brain cells have high metabolic activities and significant antioxidant defenses as inferred from the high contents

of carotenoids and polyunsaturated fatty acids, which are targets of lipid peroxidation (Barja, 2004; Agostinho et al., 2010; Johnson et al., 2013). The importance of antioxidant defense is confirmed in studies of cancer radiotherapy, where the additional intake of antioxidants decreased the effectiveness of radiation treatment (Lawenda et al., 2008).

Our results also suggest that there might be priority of protection of some biological matrices at the cost for others. For example, we found that radiation caused increased antioxidant levels in sperm, resulting in a reduction of oxidative damage. This might indicate that, when exposed to chronic LDIR, males invest more in protection of sperm in order to limit reduction in fertility and so in the ability to successfully reproduce. Interestingly, previous studies of sperm motility in barn swallows showed that the relationship with individual oxidative status depended on environmental radiation, suggesting individuals prioritize protection of sperm when exposed to LDIR, at the expense of their plasma oxidative status (Bonisoli-Alquati et al., 2011). The effect for eggs was opposite to that for sperm. It is possible that eggs and sperm differ in susceptibility to LDIR, and that this affects differences in the level of investment in antioxidant protection under LDIR.

Species varied significantly in level of antioxidant response and oxidative damage. Three species (*Myodes rutilus*, *Microtus arvalis* and *H. rustica*) showed an increase in antioxidant defenses after exposure to ionizing radiation, while three others (*Mus musculus*, *Parus major* and *Homo sapiens*) showed a decrease. Of the seven species assessed for oxidative damage levels, the four species *A. agrarius*, *A. uralensis*, *B. taurus* and *H. sapiens* showed an increase in oxidative markers, while *R. norvegicus*, *M. arvalis* and *M. rutilus* did not differ significantly from zero (Fig. 4). However, the analysis of absolute values



demonstrated that mean effect size for *Rattus norvegicus* consisted of significant positive and significant negative effects of oxidative damage. This analysis also showed that *A. sylvaticus*, *A. uralensis* and *M. glareolus* had none to very weak effects (Fig. 4). Such heterogeneity may indicate that some species are negatively impacted by ionizing radiation while others are more resistant.

Variation in effect among biological matrices within species can obscure the analysis of relationships, as seen in *R. norvegicus* and *H. rustica* (Fig. 4). While showing a non-significant level of signed oxidative effect, the average absolute effect size for these species was significantly large. Thus, we suggest care when considering the particular marker analyzed and the predicted direction of effects when combining studies that are not homogeneous in the protocol used for analyses.

The literature sources that we used in the present study reported various measurements of LDIR on human or animal populations. For example, Souchkevitch et al. (1997) and Ovsyannikova et al. (2010) reported individual effective dose of exposure in milliSieverts (mSv), whereas Paranich et al. (1998), Moroz et al. (1998) and Lyashenko et al. (2000) used centigray (cGy) as a unit of absorbed dose, and Verhoglyad et al. (1991) reported an older unit, millirem (mrem). Yet these units are cross-convertible with some minor assumptions (Thaul and O'Maonaigh, 1999), while others provide a vague estimate of individual radiation impact, reporting spatial contamination or rather the equivalent dose rate. Among such studies were those by Shishkina et al. (2005), with a commonly reported exposure rate in  $\mu\text{Sv/h}$ . Likewise, Belov et al. (1997) estimated radionuclide intake in Bq/day, while other studies like Mirzoev et al. (1999) or Neyfakh et al. (1998a) reported terrestrial contamination in  $\text{kBq/m}^2$  or  $\text{Ci/km}^2$ , respectively. Thus, because of

methodological inconsistencies in reporting radiation dosimetry among studies, we were unable to infer dose-response relationships to explain variation. Therefore, we call for the use of estimates of acquired dose of LDIR by individuals rather than reporting measurements of environmental contamination.

Young age classes are considered to be more susceptible to oxidative damage because juveniles produce large amounts of free radicals as a consequence of their development and growth process, whilst they have an enzymatic antioxidant machinery that takes time to become fully mature (Costantini, 2014; Surai, 2002). In addition, low levels of dietary antioxidants deposited by mothers into eggs or passed to the offspring through milk may result in embryos exposed to LDIR starting their development with low levels of antioxidants (Møller et al., 2005). Thus, we expected that studies of early developmental stages would demonstrate greater sensitivity to increased oxidative damage. Levels of natural LDIR vary considerably with studies in high natural background radiation areas finding weak, but significant health effects associated with natural LDIR (Møller and Mousseau, 2013). This conclusion suggests that there is selection acting on the ability to sustain LDIR, and that there may be scope for adaptation to radiation. Indeed, studies of birds at Chernobyl and Fukushima have shown that species with carotenoid- and pheomelanin-based pigments in their plumage show stronger negative impact of LDIR on population density (Galván et al., 2011; Møller and Mousseau, 2009, 2007b; Møller et al., 2012). At the same time, Galván et al. (2014) showed that glutathione (an important intracellular antioxidant) levels and body condition increased, and oxidative damage and DNA damage decreased, with increasing background radiation in some species of birds. This effect was independent of a number of potentially confounding variables including

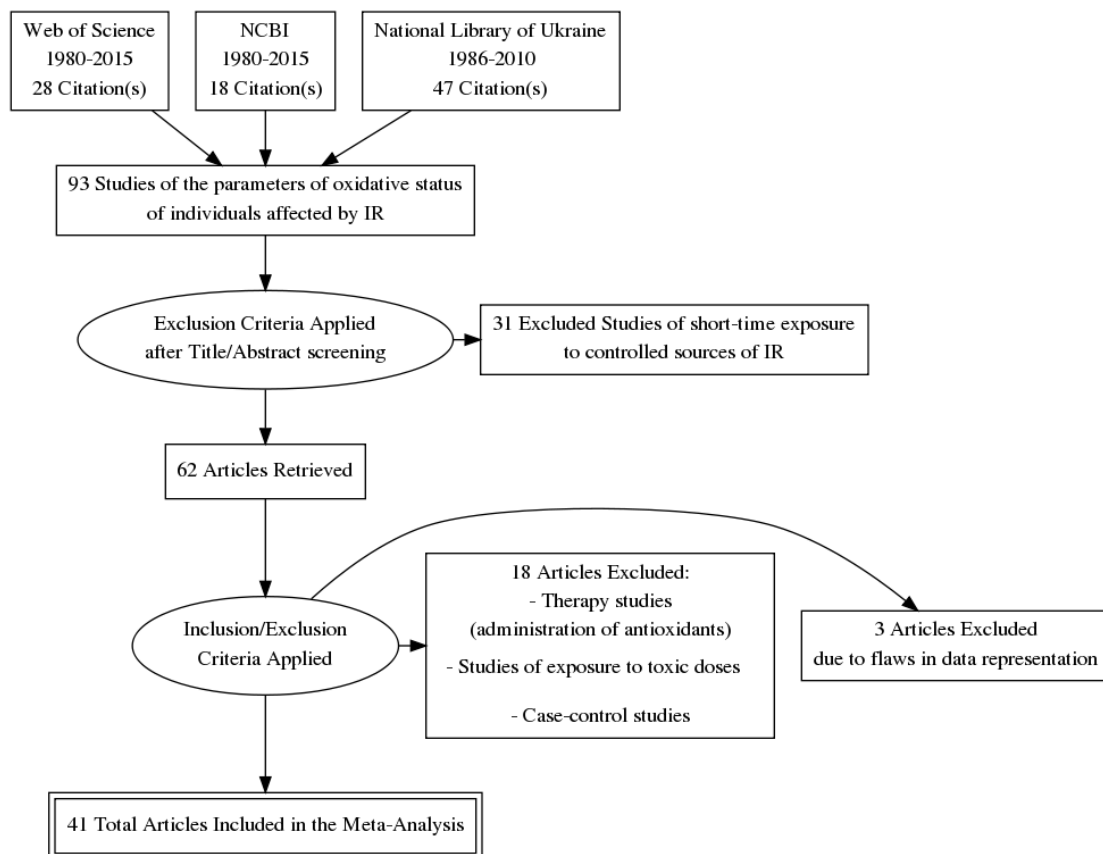
effects of similarity among taxa due to common phylogenetic descent. We only found a significant difference in the effect of LDIR on oxidative damage among species and age classes in a meta-regression accounting for the effects of other potentially confounding variables. In contrast, there was a significant effect of species and matrix on the level of antioxidant response. While some species showed a positive response for oxidative damage, other species did not. This dichotomy and the significant heterogeneity among species are consistent with adaptation to LDIR although alternative explanations may also account for such heterogeneity (Lademann et al., 2015).

In conclusion, we have found generally strong effects on oxidative status in response to low-dose ionizing radiation. Meta-analyses weighted by sample size showed significant heterogeneity among biological matrices, species and age classes. These findings are consistent with some species apparently being negatively impacted by ionizing radiation while others are not, or even showing evidence consistent with adaptation to radiation by having positive antioxidant responses at high levels of radiation.

The results of our meta-analysis have important implications for studying the effects of LDIR in human populations and in the wild. For example, over the last few decades exposure of the average American to ionizing radiation has increased from 3.6 to 6.2 mSv per year (NCRP, 1987, 2009). Generally, this low rate of exposure comes from natural sources in air, soil, rocks and cosmic rays (accounting for 2-2.5 mSv per year), while the remainder is acquired from man-made sources such as medical procedures (periodic X-ray, CT scans and others) and industrial activities such as those associated with mining and processing of ores, minerals, nuclear fuels and oil. Such trends as those revealed in the NCRP reports and elsewhere forecast an increase in exposure due to such

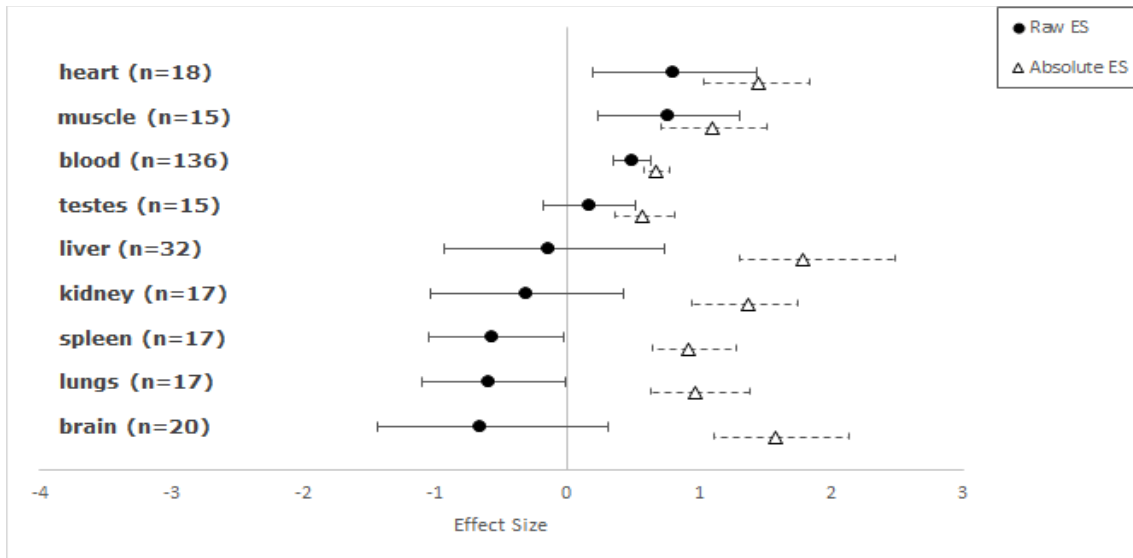
artificial sources (Fazel et al., 2009; Pandey et al., 2010). In addition, the advent of the “Atomic age” has ushered in several nuclear accidents. Two of these, at Chernobyl NPP in 1986 and at Fukushima NPP in 2011, were classified at the highest level on the International Nuclear and Radiological Event Scale (INES) scale of nuclear hazards leading to global increases in radioactive contamination. Overall, man-made sources of LDIR appear to increase radioactive intake, which, as our analysis suggests, might be reflected in the metabolic sensitivity to LDIR.

## FIGURES AND TABLES

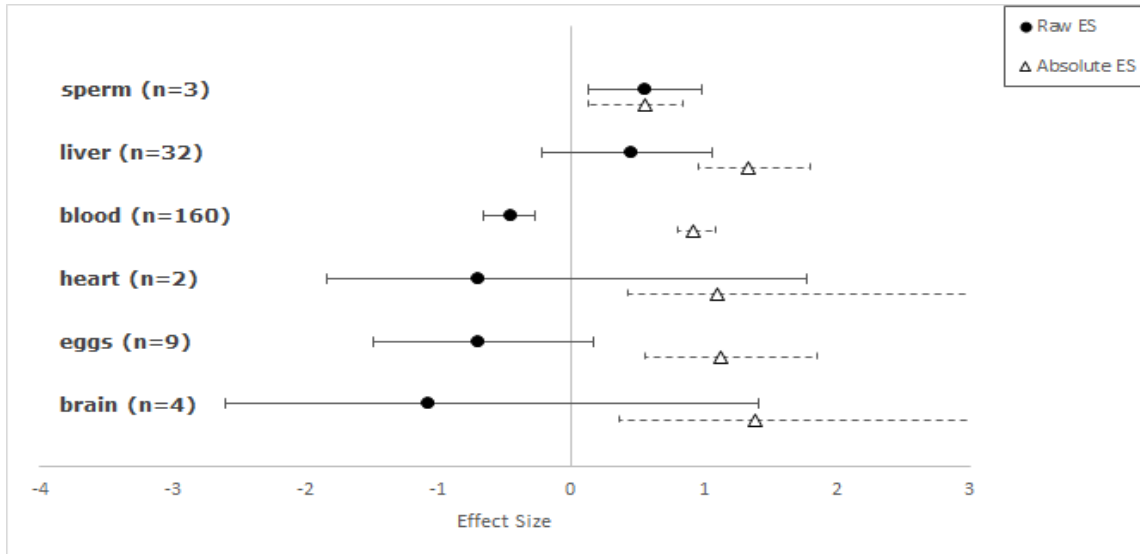


**Figure 1.1** The PRISMA flow diagram of the initial publications and the subsequent number of papers retained for the meta-analysis.<sup>2</sup>

<sup>2</sup> <http://prisma.thetacollaborative.ca/>

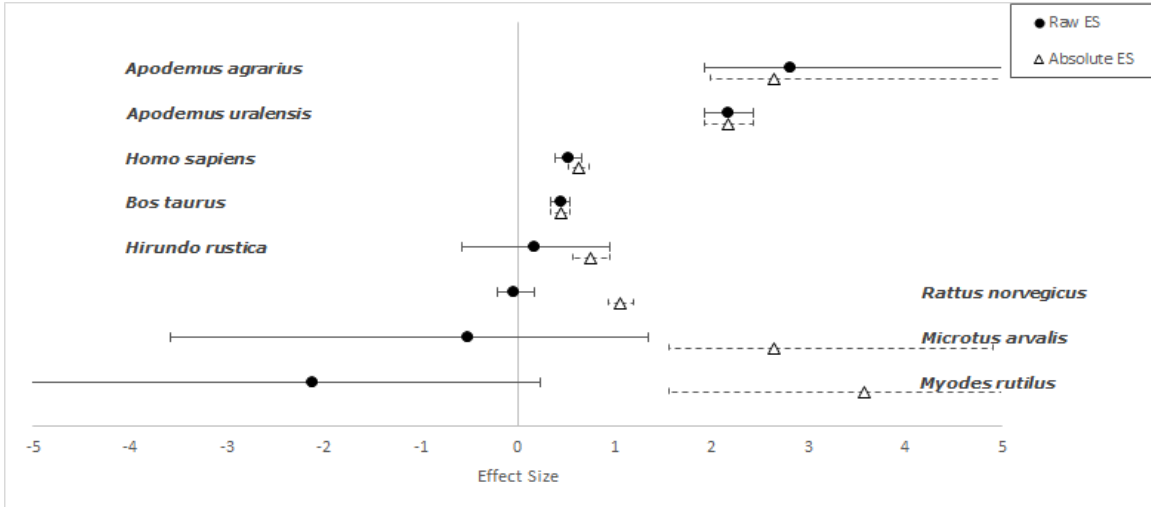


**Figure 1.2** Forest plot for mean and absolute effect sizes<sup>3</sup> of oxidative damage in biological tissues.

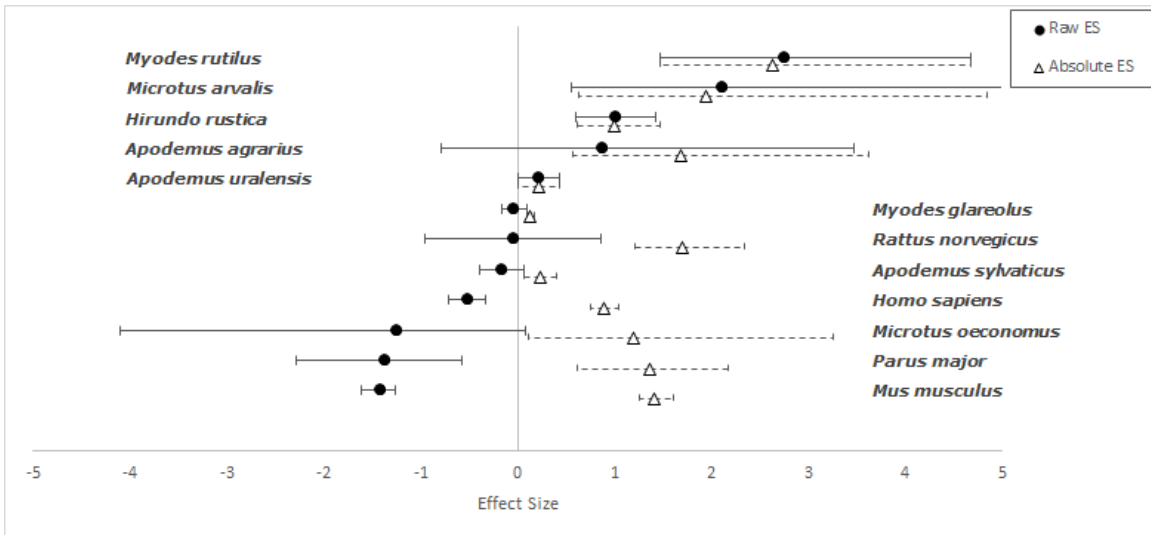


**Figure 1.3** Forest plot for mean and absolute effect sizes of antioxidant response in biological tissues.

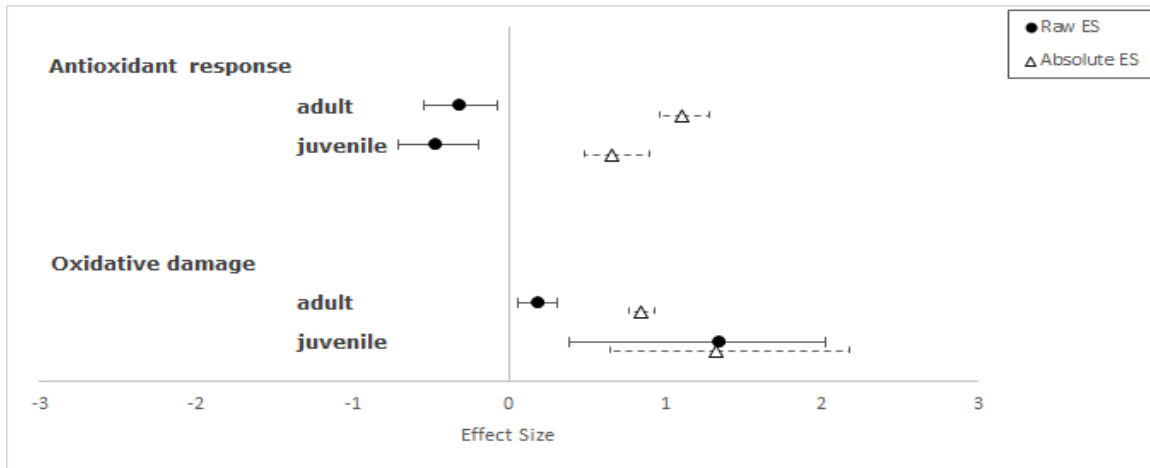
<sup>3</sup> Here and in all following forest plots projected values are means and 95% bootstrap confidence intervals



**Figure 1.4** Forest plots for mean and absolute effect sizes of oxidative damage for different species.



**Figure 1.5** Forest plots for mean and absolute levels of antioxidant response for different species.



**Figure 1.6** Forest plots for levels of mean and absolute values of antioxidant response and oxidative damage for the two age classes.

**Table 1.1** Signed effect size estimates for antioxidant response and oxidative damage weighted by sample size.<sup>4</sup>

	Effect size	N	Lower B-strap CI	Upper B-strap CI	$Q_{Total}$	$P$	Rosenthal's failsafe number
<b>Antioxidant response</b>	<b>-0.35</b>	212	<b>-0.53</b>	<b>-0.17</b>	<b>454.3</b> <b>3</b>	<b>0.001</b>	<b>1698.2</b>
<b>Oxidative damage</b>	<b>0.276</b>	288	<b>0.086</b>	<b>0.37</b>	<b>735.8</b> <b>8</b>	<b>0.001</b>	<b>708.7</b>

<sup>4</sup> Table also reports sample size, lower and upper 95% bootstrap confidence intervals, test for heterogeneity ( $Q_{Total}$ ),  $P$ -value for heterogeneity test and Rosenthal's failsafe number. Herein through Chapter 1 signed effect sizes shown in bold are significantly ( $P < 0.05$ ) different from zero



**Table 1.2<sup>5</sup>** Signed and unsigned effect size estimates weighted by sample size for antioxidant response and oxidative damage in biological tissues.

	Effect Size (signed)	N	Lower b-strap CI	Upper b-strap CI	Unsigned Effect Size	Lower b-strap CI	Upper b-strap CI
Antioxidant response by biological tissue							
<b>blood</b>	<b>-0.4558</b>	<b>160</b>	<b>-0.6467</b>	<b>-0.2536</b>	<b>0.9237</b>	<b>0.7974</b>	<b>1.0871</b>
liver	0.4574	32	-0.1518	1.1338	<b>1.3373</b>	<b>0.9593</b>	<b>1.7977</b>
brain	-1.0769	4	-3.5724	0.4507	<b>1.3899</b>	<b>0.3616</b>	<b>4.2475</b>
heart	-0.7009	2	-3.171	0.4337	<b>1.1017</b>	<b>0.4337</b>	<b>3.171</b>
eggs	-0.7048	9	-1.5757	0.0749	<b>1.1296</b>	<b>0.5569</b>	<b>1.8472</b>
<b>sperm</b>	<b>0.5558</b>	<b>3</b>	<b>0.1251</b>	<b>0.9857</b>	<b>0.5554</b>	<b>0.1251</b>	<b>0.8428</b>
Oxidative damage by biological tissue							
<b>blood</b>	<b>0.4936</b>	<b>136</b>	<b>0.3533</b>	<b>0.6328</b>	<b>0.6705</b>	<b>0.5856</b>	<b>0.778</b>
liver	-0.1426	32	-1.0279	0.6498	<b>1.7837</b>	<b>1.3136</b>	<b>2.4897</b>
brain	-0.6542	20	-1.6221	0.134	<b>1.5866</b>	<b>1.118</b>	<b>2.1366</b>
<b>lungs</b>	<b>-0.5896</b>	<b>17</b>	<b>-1.1671</b>	<b>-0.0752</b>	<b>0.9667</b>	<b>0.6354</b>	<b>1.3833</b>
<b>heart</b>	<b>0.8035</b>	<b>18</b>	<b>0.1717</b>	<b>1.4084</b>	<b>1.4518</b>	<b>1.0392</b>	<b>1.8409</b>
<b>spleen</b>	<b>-0.5653</b>	<b>17</b>	<b>-1.1006</b>	<b>-0.0782</b>	<b>0.919</b>	<b>0.6431</b>	<b>1.2882</b>
kidney	-0.3142	17	-1.0505	0.4097	<b>1.3763</b>	<b>0.945</b>	<b>1.7523</b>
<b>muscle</b>	<b>0.764</b>	<b>15</b>	<b>0.2166</b>	<b>1.2885</b>	<b>1.1081</b>	<b>0.7184</b>	<b>1.5126</b>
testes	0.1756	15	-0.1659	0.5277	<b>0.5654</b>	<b>0.3657</b>	<b>0.8158</b>

<sup>5</sup> This and the following tables also report sample size, lower and upper 95% bootstrap confidence intervals.

**Table 1.3** Signed and unsigned effect size estimates weighted by sample size for antioxidant response and oxidative damage in different species.

	Effect Size (signed)	N	Lower b- strap CI	Upper b- strap CI	Unsigned Effect Size	Lower b- strap CI	Upper b-strap CI
Antioxidant response by species							
<i>Homo sapiens</i>	<b>-0.5179</b>	<b>149</b>	<b>-0.7161</b>	<b>-0.3265</b>	<b>0.8928</b>	<b>0.7543</b>	<b>1.0422</b>
<i>Rattus norvegicus</i>	-0.0369	19	-0.9505	0.8549	<b>1.6966</b>	<b>1.2086</b>	<b>2.3345</b>
<i>Hirundo rustica</i>	<b>1.0128</b>	<b>11</b>	<b>0.6021</b>	<b>1.4296</b>	<b>1.0003</b>	<b>0.6147</b>	<b>1.4664</b>
<i>Parus major</i>	<b>-1.3696</b>	<b>6</b>	<b>-2.2902</b>	<b>-0.5828</b>	<b>1.3562</b>	<b>0.6100</b>	<b>2.1749</b>
<i>Apodemus agrarius</i>	0.8725	5	-0.7928	3.4625	<b>1.6896</b>	<b>0.5651</b>	<b>3.6249</b>
<i>Mus musculus</i>	<b>-1.413</b>	<b>5</b>	<b>-1.616</b>	<b>-1.2564</b>	<b>1.4121</b>	<b>1.2616</b>	<b>1.6065</b>
<i>Microtus oeconomus</i>	-1.2427	4	-4.1036	0.0749	<b>1.1941</b>	<b>0.1109</b>	<b>3.2536</b>
<i>Microtus arvalis</i>	<b>2.1033</b>	<b>4</b>	<b>0.5600</b>	<b>5.0518</b>	<b>1.9357</b>	<b>0.6265</b>	<b>4.8408</b>
<i>Myodes rutilus</i>	2.7442	2	1.4713	4.6699	2.6359	<b>1.4713</b>	<b>4.6699</b>
<i>Apodemus uralensis</i>	0.2167	2	0	0.4337	0.2166	0	0.4337
<i>Apodemus sylvaticus</i>	-0.1701	2	-0.3994	0.0588	0.2289	<b>0.0588</b>	<b>0.3994</b>
<i>Myodes glareolus</i>	-0.0366	2	-0.1693	0.0961	0.1327	<b>0.0961</b>	<b>0.1693</b>
Oxidative damage by species							
<i>Rattus norvegicus</i>	<b>-0.0428</b>	<b>155</b>	<b>-0.2114</b>	0.1690	1.0594	0.934	1.1925
<i>Homo sapiens</i>	<b>0.523</b>	<b>110</b>	<b>0.3922</b>	0.6629	0.6271	0.5265	0.7334
<i>Microtus arvalis</i>	-0.5189	9	-3.5842	1.3470	2.6492	1.5616	4.9091
<i>Bos taurus</i>	<b>0.4457</b>	<b>3</b>	<b>0.3406</b>	<b>2.4855</b>	0.4456	0.3406	0.5452
<i>Myodes rutilus</i>	-2.1144	3	-7.4000	0.2310	3.5682	1.5603	7.4
<i>Apodemus agrarius</i>	2.8164	3	1.9291	8.7224	2.6476	1.9937	8.7224
<i>Apodemus uralensis</i>	<b>2.1756</b>	<b>2</b>	<b>1.9291</b>	<b>2.4273</b>	2.1736	1.9291	2.4273
<i>Hirundo rustica</i>	0.1737	2	-0.5711	0.9516	0.7541	0.5711	0.9516

**Table 1.4** Signed and unsigned effect size estimates for antioxidant response and oxidative damage in age groups weighted by sample size

	Effect Size (signed)	N	Lower b-strap CI	Upper b-strap CI	Unsigned Effect Size	Lower b-strap CI	Upper b-strap CI
Antioxidant response by age							
<b>adult</b>	<b>-0.315</b>	<b>155</b>	<b>-0.5616</b>	<b>-0.0822</b>	<b>1.1109</b>	<b>0.9627</b>	<b>1.2776</b>
<b>juvenile</b>	<b>-0.4668</b>	<b>55</b>	<b>-0.7373</b>	<b>-0.2288</b>	<b>0.6585</b>	<b>0.4831</b>	<b>0.9051</b>
Oxidative damage by age							
<b>adult</b>	<b>0.1866</b>	<b>275</b>	<b>0.0586</b>	<b>0.3156</b>	<b>0.8481</b>	<b>0.769</b>	<b>0.9351</b>
<b>juvenile</b>	<b>1.343</b>	<b>11</b>	<b>0.6638</b>	<b>2.3029</b>	<b>1.3226</b>	<b>0.6534</b>	<b>2.1804</b>

**Table 1.5** Ordinary least squares model of effect size<sup>6</sup> of oxidative damage weighted by sample size in relation to species, tissue type and age.

Term	<i>df</i>	Sum of squares	<i>F</i>	<i>P</i>	Estimate	SE
Intercept			0.212	0.646	0.286	0.624
Species	6	2173.44	2.344	0.032		
Tissue type	9	1377.95	0.991	0.448		
Age	1	843.14	5.46	0.020	-0.493	0.211
Error	252	38947.77				

**Table 1.6** Ordinary least squares model of effect size<sup>7</sup> for antioxidant response weighted by sample size in relation to species, tissue type and age.

Term	<i>df</i>	Sum of squares	<i>F</i>	<i>P</i>	Estimate	SE
Intercept			7.182	0.008	-2.660	0.992
Species	10	3578.30	2.449	0.009		
Tissue type	7	3552.67	3.484	0.0016		
Age	1	724.86	4.975	0.027	-0.269	0.121
Error	177	25787.78				

<sup>6</sup> The model had the following statistics:  $F = 2.413$ ,  $df = 16, 252$ ,  $r^2 = 0.08$ ,  $P < 0.0022$ .

<sup>7</sup> The model had the following statistics:  $F = 3.9151$ ,  $df = 18, 177$ ,  $r^2 = 0.17$ ,  $P < 0.0001$ .

## ACKNOWLEDGMENTS

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## CHAPTER 2.

### DNA DAMAGE, IMMUNE STATUS AND BODY BURDEN OF CESIUM-137 IN THE BANK VOLES (*MYODES GLAREOLUS*) OF CHERNOBYL<sup>8</sup>

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## ABSTRACT

In this study, wild bank voles *Myodes glareolus* that inhabit the Exclusion zone around the Chernobyl Nuclear Power Plant were investigated for the levels of DNA fragmentation (using the comet assay) and relative concentration of the nucleated cells in blood and the whole-body burden of radioactive cesium-137. To predict the blood parameters, we constructed mixed-effects models that included radioactivity data, sex and age categories of animals, and we also accounted for sampling location and experimental sequence as random effects. Our results showed non-significant positive association of comet DNA damage with both radiation estimates: body burden of cesium, and environmental exposure rate, suggesting little to no radiation effects on single- and double-stranded breaks in voles' white blood cells. Instead, a significant effect of sex was found: on average, female voles tended to show less DNA damage, but males showed a stronger, albeit non-significant, relationship between damage and radiation. Also, female voles possessed significantly more nucleated cells than males, but males showed a stronger yet non-significant increase in cell count under increasing radioactivity.

## INTRODUCTION

Systematic analyses of radiotoxicological studies overwhelmingly support the fact that ionizing radiation (IR) can bring about strong biological effects (e.g., reviews: Yablokov *et al.*, 2009; Little, 2016; Lourenço, *et al.* 2016; meta-analyses: Møller & Mousseau, 2014; Einor *et al.* 2016). Overall these studies indicate that elevated ionization (including low-dose IR, LDIR) is a driver of disturbance in biological systems. Notwithstanding the ability of tissues, organs, and their systems to repair or tolerate the damage from IR, the intrinsic molecular effects of radiation may lead to substantial long-term genetic and physiological consequences (Spitz, *et al.*, 2004; Tharmalingam, *et al.*, 2017, Tang, *et al.*, 2017). In fact, the evidence that the mutation process is generally positively associated with exposure to IR has been known for almost a century (Muller & Altenburg, 1930). In addition, IR was found to induce chemically active radicals (such as the reactive oxygen species, ROS), which affect the stability of the genetic machinery, comprised of DNA and its associated proteins, as well as cellular complexes and lipid membranes (Aitken & Krausz, 2001; Itsara, *et al.*, 2014). The mechanism of radiation syndrome is proposed as a cumulative aggravation of these effects, which would then drastically reduce individual fitness. (Dörr & Meineke, 2011). The most extreme cases are typically seen under conditions of high acute exposures. At the same time, the toxic effects of LDIR are much less apparent, and the consequences of LDIR exposure are much less investigated. (Møller & Mousseau, 2013). This issue is of a great interest for the scientific community and general public, as human exposure to IR is increasing due to medical procedures, technological advancements and collateral contamination related to energy production via nuclear power plants (NCRP, 2009; Mousseau & Møller, 2017).



In the Chernobyl Exclusion Zone (CEZ), which is similar to other places contaminated with scattered products of the nuclear cycle (e.g. Fukushima prefecture, Japan; Mayak, Russia; Sellafield, UK), the main external exposure from the environment comes in the form of gamma-rays which are penetrative enough to reach the body from the soil surface. In the CEZ, cesium-137 is the major contributor to gamma radiation (UNSCEAR, 2014). Cesium generally mimics potassium in biological systems which results in its bio-circulation properties, and thus its long-living isotope ( $^{137}\text{Cs}$ ,  $T_{1/2} = 30,16$  a) is one of the main biomedical concerns in nuclear waste management. In the field, rates of gamma-exposure are simple to estimate using a Geiger counter. In the case of the CEZ this allows for relative approximation of environmental contamination by cesium, as it is one of the remaining (along with beta-emitting strontium-90) most abundant radioisotopes. Under certain assumptions, these rates may be then converted into cumulative dose estimates that an organism received (Ramola *et al.*, 2016; dos Santos Júnior *et al.*, 2017). Once ingested through food, air or water, radioactive elements can deliver more intense IR, with the additive effect from the exposure to beta- and alpha—particles (e.g. from Plutonium-239 and its short-living daughter isotopes, along with strontium-90 and cesium-137) (Florou *et al.* 2010; Harada *et al.* 2013). Additional accounting for the ingested and incorporated radionuclides through measurements of the body burden of radioactivity is essential in providing more precise information about the radiation dose (UNSCEAR, 2014). Finally, proper reconstruction of the absorbed dose also requires knowledge about the levels of soil contamination by radionuclides, which sometimes can be difficult to estimate because of the costs associated with radiochemical analysis of mineral or biological samples (Ulanovsky & Pröhl, 2012; Modorov *et al.*, 2017). The absorbed dose

is derived with account of all effective radioisotopes in the system, for which the dose conversion coefficients (DCC) given internal or external contamination and the size type of an animal have been previously estimated. (Taranenko, *et al*, 2004; Ulanovsky & Pröhl, 2012; review in Stark, *et al*, 2017). The stochastic risk of the overall health effect is proposed to come from the effective dose – estimate that accounts for the relative radiosensitivity of the target biological tissues together with the most accurate estimate of the total absorbed dose (ICRP, 2009).

Small mammals such as rodents, because of their “close proximity” to the contaminants in the soil and short generation turn-over, present a convenient ecological model to study the population effects of LDIR. Notably, a significant heterogeneity of radiation effects was demonstrated for wild murid species (e.g. mice, rats, voles, hamsters etc.), in contrast to what was historically observed for the murine model systems under a controlled environment. For example, laboratory rodents, such as house mice (*Mus musculus*) and laboratory rats (*Rattus norvegicus*) are known for their sensitivity to Chernobyl-like rates of LDIR, which can be inferred from the elevated mutation rates (Okudaira *et al.*, 2010), organ failure (El-Shanshoury, *et al.*, 2016; Bellés, *et al.*, 2017) and reduced fertility (Jagetia & Krishnamurthy, 1995). In comparison, their wild murid relatives from the *Myodes* genus (voles) can be observed in areas with relatively high radiation conditions. Some individuals were found to accumulate much higher levels of radioactive cesium compared to the close-related murids, such as meadow voles *Microtus*, or phylogenetically distant species of *Apodemus* (e.g. wood mice), suggesting significant variation in survival due to variation in radiosensitivity across these species (Chesser, *et al.*, 2000; 2001). In terms of somatic effects, some studies found evidence of decreased

health status, as seen from the elevated incidence of radiation-associated cataract (Lehmann, *et al.*, 2016), increased fluctuating asymmetry (Oleksyk, *et al.*, 2004) and slowly recovering populations (e.g. *Apodemus* and *Myodes* of the EURT, Grigorkina, *et al.* 2006) of the wild rodents. Analyses of genotoxicity associated with environmental contamination suggests that wild rodent species express more frequent micronuclei in blood cells (Grigorkina, *et al.* 2007) and a general increase in chromosomal anomalies (Kudryasheva, *et al.*, 2007; Yalkovskaya, *et al.*, 2011). Conversely, some studies failed to detect the expected effects on conventional radiation biomarkers such as oxidative stress (Ustinova & Ryabinin, 2003), allozyme variation (Modorov, 2009), genetic damage or mutation rate (Rodgers *et al.*, 2001; Wickliffe, *et al.* 2002). Such inconsistency in the radioecological literature emphasizes the importance of further studying natural responses to the chronic stress caused by IR, especially in ecosystems that were affected by nuclear accidents, because such studies could provide a better understanding of the physiological response to LDIR in humans (Galván, *et al.* 2014; Møller & Mousseau, 2016).

In this report, we present the results of genotoxicity testing of Chernobyl populations of bank voles *Myodes glareolus* and its correlation to the severity of radioactive contamination, as measured both externally and internally. We used the comet assay method (Olive, *et al.*, 2009), because it straightforwardly visualizes the magnitude of DNA breakage by the shapes of the assessed cell nucleoids. The classic alkaline comet assay protocol for blood PBMC by Singh (1988) and its simplified version by Olive & Banáth (2006) was successfully applied for radiation studies on humans (e.g. Baumgartner, *et al.*, 2009), laboratory-bred and wild animals, including birds (Bonisoli-Alquati, *et al.*, 2010), amphibians (Valencia, *et al.*, 2011), fish (Pereira, *et al.*, 2011), and invertebrates

including mussels (Alamri, *et al.*, 2012) and insects (Hasan, 2006). It has been shown that the DNA lesions which are commonly detected by the comet assay, may ultimately lead to chromosome breakage and are associated with major translocation events (Iliakis, 1991; Fairbairn, *et al.*, 1995). The method involves fixation of the blood sample in agarose and then undergoes incubation with different agents. The primary lysis buffer contains an amphiphilic surfactant (Triton-X100), chelating agent (EDTA-Na<sub>2</sub>) and sodium salt (NaCl). This treatment causes lysis of cellular membranes, inhibition of enzyme activity (specifically endo- and exonucleases), and most importantly - electrostatic dissociation and disentanglement of the DNA-histone complexes that form the nucleosomes (Stein & Page, 1980). In most cases of radiation-damage response studies, a positive dose-response relationship was discovered. Therefore, we also predicted that levels of DNA damage would linearly increase with the increase of estimates of the environmental and internal radioactivity. Alternatively, because a very low response to oxidative stress (Ustinova & Ryabinin, 2003; Einor *et al.* 2016) and very high radiation burden were previously observed in *M. glareolus* (Chesser, *et al.*, 2001), we suggested that genetic damage as measured by the comet assay in these species might not show an apparent effect of radiation.

A negative effect of aging on the damage repair of DNA has been demonstrated by multiple studies (e.g. Ju, *et al.*, 2006; Garm, *et al.* 2013), therefore we also expected that older voles would express higher levels of comet DNA fragmentation. In addition, some studies have suggested gender-associated variation in the activity of DNA repair, and hence, differences in genetic damage between males and females could be expected. (Trzeciak, *et al.*, 2008). For example, Slyskova *et al.* (2011) reported elevated DNA

damage in human males in response to a DNA-damaging agent. Higher rates of genetic damage were also found in human males in a different study (Hofer, *et al.*, 2006) of DNA damage related to oxidative stress. Therefore, to test for the possible effect of sex, we included gender information of the voles into our predictions of genetic damage.

Additionally, we evaluated the immune status of animals by approximating the concentration of white blood cells (WBC) in whole blood samples from the comet assay results. We predicted that bank voles from contaminated areas would be characterized by depressed immune status, similar to the radiation-associated decrease in WBC counts in experimental studies (Boghdándi, *et al.*, 2010; El-Shanshoury, *et al.*, 2016). We also predicted age-dependent decline in immune function, as it was observed in previous studies (Gill, *et al.*, 2001). Therefore, we expected decreased WBC counts in older voles.

The aim of the present study was to provide further insights for understanding biological responses to radioactive contamination, with a specific goal of investigating mutagenic effects of environmental and incorporated radioactivity on natural, free-living mammalian populations (Møller & Mousseau, 2016; Bréchignac *et al.*, 2016).

## MATERIALS AND METHODS

### *Sampling*

Wild bank voles, *Myodes glareolus*, were captured from 40 locations with varying levels of radioactive contamination within three major areas of the Chernobyl Exclusion Zone (CEZ) (Fig. 2.1): (1) the northern part of the CEZ (Chernobyl North, CN), which received main fallout after the reactor 4 of the ChNPP failed, and had the highest levels of contamination, contained 22 replicate trapping sites; (2) the south-western part of the CEZ

(near Rassokha village, Chernobyl South-West, CSW) with relatively low ambient radiation levels, containing 13 replicate sites; (3) the south-eastern part of the CEZ (near the city of Chernobyl, Chernobyl South-East, CSE) which is characterized by near-normal exposure rates, and contained five replicate sites. The sites (2) and (3) were control sites based on previous records of the areas that were not significantly affected by the fallout (Ageev, *et al.*, 1995).

Animals were trapped during May-June of 2014 (N=108; 62 males; 46 females) and 2015 (N=283; 193 males; 90 females), and in September-October of 2014 (N=414; 200 males; 214 females). Briefly at each location, 9-16 potato-baited Ugglan live traps were placed in a 3x3 or 4x4 grid with a 10m interval between traps and were checked every morning after each of three consecutive nights. Captured animals were transferred to a laboratory at Chernobyl city and placed into individual Makrolon Type III cages (43x26x15cm). Head width, weight and body length were measured for all individuals and a sub-set of voles was examined for gravidity and presence of tumors upon sacrifice. All procedures were performed in accordance with international guidelines and regulations for the use of animals in research. The study was approved by the Finnish Ethical Committee (license number ESAVI/7256/04.10.07/2014).

### *Dosimetry*

Ambient environmental radioactivity within the CEZ was measured using pre-calibrated handheld dosimeters (Inspector, International Medcom, Inc., Sebastopol, CA, USA) at 1cm above the ground-level. Measurements were taken several times and then averaged for a point estimate in  $\mu\text{Sv h}^{-1}$ , which was the default unit of measurement for

this dosimeter. Range and mean exposure rates at the trapping locations were as follows: (1) CH sites: range 1.69– 136.72  $\mu\text{Sv h}^{-1}$  (mean = 32.96  $\mu\text{Sv h}^{-1}$ ); (2) CSW sites: range 0.12 – 0.50  $\mu\text{Sv h}^{-1}$  (mean = 0.13  $\mu\text{Sv h}^{-1}$ ); (3) at CSE sites: range 0.14 – 0.36  $\mu\text{Sv h}^{-1}$  (mean = 0.17  $\mu\text{Sv h}^{-1}$ ). Overall, this range ensured an approximately 10 to 1000-fold difference in exposure rates between the control and experimental sites.

Earlier studies have reported that the biological half-lives of radioisotopes can vary significantly depending on their bioaccumulation properties (Chesser, *et al.* 2001; Reichle, *et al.* 1970). Our experimental analyses for bank voles suggested a biological half-life of  $^{137}\text{Cs}$  of approximately 100 hours (Fig 2.2), presumably due to excretion. To control for the excretion loss, we measured the body burdens around 12-20 hours after animal trapping. All body burden measurements were conducted using a Berkeley Nucleonics SAM940 radionuclide identifier system equipped with a 10x10x10 cm NaI detector housed in lead shielding, which minimized the background radioactivity to about 7.5 gamma counts per second (GCPS). The system was pre-calibrated using a 0.204  $\mu\text{Ci}$  source, which allowed us to generate a calibration factor used to convert the counts from the tested samples to Ci. The geometrical parameters of the source were similar to the tested samples. Exposure time was set between 5 and 15 minutes per individual vole, depending on the prospected activity levels with higher activities requiring shorter reading times. Obtained spectra were compared with a background reference spectrum. Activity of  $^{137}\text{Cs}$  was estimated by integrating the activity above the background threshold at 662 keV, which is the primary energy level for cesium-137 decay products. Subsequently the radioactivity measurements were standardized across samples by individual mass, measured on a Mettler electronic balance directly after spectrum evaluation.

### *Blood Collection and Storage*

Blood samples from live animals were collected via a retro-orbital bleed with heparin-coated capillaries (at least 50  $\mu\text{l}$  per individual). Upon collection, the blood from capillaries was transferred to tubes containing 450  $\mu\text{l}$  of RNAProtect® (QIAGEN) preservative and then the whole solution was gently resuspended. The tubes were then immediately cooled to +4°C before being transferred to the University of South Carolina, Columbia, for further processing and cryopreservation

### *The Comet Assay:*

Slides for the comet assay were precoated with 1% regular laboratory-grade agarose solution at least one day before assay. Eppendorf® tubes with 200  $\mu\text{l}$  1% low melting point agarose (LMPA) in 1x PBS solution were freshly prepared for each experimental day. Tubes were maintained at 38°C when the aliquot of a blood samples was resuspended in the solution. We applied a combined lysis that involved preliminary enzymatic treatment that was followed by a conventional comet assay procedure (following Singh *et al.* 1988, and Olive & Banáth, 2006, with similar modification as in Su & Song, 2008). Each 20 $\mu\text{L}$  aliquot of blood-in-RNAProtect® was gently resuspended and mixed in the LMPA solution in 1:10 proportion, to which 20  $\mu\text{L}$  of a Proteinase K working solution (Lysis buffer #1, “LB1” – 2.5 $\mu\text{g}$  of Proteinase K; PK buffer: 0.1M Tris-Cl, pH 7.5; 0.2M NaCl; 0.002M EDTA; 1% Triton-X100) was immediately added. The tubes were incubated in a dry thermostat at 38°C for 10 minutes and then the mixture was pipetted onto the slides. For each sample suspension, two replicates were made with opposite positioning of the mixture droplet on each slide. Each droplet was immediately covered with a 22x22 mm coverslip



and batches of 20 slides (20 samples) were prepared for each electrophoretic run. After coverslips were placed, the batch was moved to a dark incubator at 4°C and slides were briefly stored to allow the blood-agarose mixture to solidify. Following this, the glass coverslips were carefully removed and the slides were placed into vertical glass staining jars containing Lysis buffer #2 (“LB2” – 2.5M NaCl, 0.1M EDTA, 0.01M Tris, pH 10.01 with freshly prepared 1% Triton-X100 and 2% DMSO) and left overnight at 4°C in a refrigerator. The staining jars were gently shaken after 2 hours of lysis to allow even diffusion of the buffer. All further steps were conducted under dim light conditions using iced buffers to minimize potential experimental error.

*Electrophoresis conditions:*

After overnight incubation, the slides were removed from the LB2 and gently washed with alkaline buffer (AB – 0.2M NaOH, 0.001M EDTA, pH>12) and placed into an electrophoretic rig containing the same AB solution, which was continuously recirculating. The timer was started when the batch was exposed to the AB solution and was set for 10 minutes. Slides were placed into an electrophoresis rig in a haphazard manner, at least 1cm away from the sides, and aligned towards the anode. Following 10 minutes to stabilize, an electric current was applied at 0.7V/cm for 10 minutes. Following that, the slides were quickly transferred to a neutralizing solution (NB) (0.2M Tris Cl pH 7.49) and were rinsed three times for 5 minutes with a fresh NB solution. The slides were then fixed in 75% ethanol for 15 minutes and allowed to fully dry before staining.

In addition, we confirm and second the statement published by Collins, *et al.* (2008), that the voltage and distance between electrodes (or the voltage-to-distance ratio) must be reported in the methods sections of a research paper on the comet assay. The voltage-to-distance ratio (V/cm) defines the electric field and a stand-alone reporting of a current provides little information about the electrophoresis conditions. The current is defined by the resistance of the buffer solution, and the latter depends on the volume of the buffer in the electrophoresis chamber. We found no difference in our preliminary analyses whenever the rig was filled within the range of 1.4-3.0L of the standard alkaline buffer, that covered the stage 0.2-1.7mm and resulted in a current of 150 to 290 mA, respectively. Furthermore, recirculation of a buffer in the chamber ensures evenness of the ions in solution and therefore promotes evenness of the electric field in multiple runs (Gutzkow, *et al.*, 2013).

#### *Staining, Scoring and Statistical analysis*

The slides were stained with SYBR® Gold Nuclear Acid Gel Stain by applying the working solution (1:30,000) onto each of the dry agarose squares on the slides and spreading with a coverslip. Slides were left for 5 minutes and then washed with deionized water and let fully dry. Nucleoids were visualized and profiled using the Metafer™4 semi-automatic comet assay system using a pre-calibrated classifier across all runs. Up to 300 comet-looking objects were captured by the camera imager per slide replicate. Ratio of the fluorescence of the migrated DNA to the total DNA (% of tail DNA) of a captured nucleoid was used as a measurement of the DNA damage. Manual validation of the comet profile was conducted for each sample to ensure that artifacts or oddly scored comets were

eliminated. Samples that contained in total less than 100 ‘comet’ objects were not included in the analysis.

The comet data were summarized for each sample (Lovell, *et al.*, 1999) and log-transformed in accordance with recommendations by Bright, et al (2011). Briefly, the median values, representing the central tendencies of a comet variation in an individual blood sample were used. The data were analyzed using simple linear regression and generalized linear mixed models using R *lme4* package (Bates, et al 2015), and SAS statistical software ver. 9.3 using PROC REG, PROC GENMOD and PROC MIXED with RANDOM statement (SAS Institute Inc., Cary, NC, USA). Figures were produced using R *ggplot2* (Wickham, 2009). The models are listed in Table 1 and Table 2. We also evaluated the association of radioactivity estimates and comet DNA damage using tumor presence as a two-level factor and female gravidity as a covariate as well as the interaction between these and other parameters included in the model. These factors did not reach statistical significance in neither of the models and thus were excluded from the analysis. We also accounted for the quality of sample preservation and subsequent comet assay methodologies by evaluating the repeatability and reproducibility of our multi-experimental set-ups.

Regression coefficients were derived from the full mixed-effects models that fitted levels of comet DNA damage and estimates of relative cell concentration as dependent variables, whereas measurements of individual body burden of cesium-137 or environmental Exposure Rate were used as fixed effects; experimental trial number and trap location were accounted as random effects. Akaike information criterion (AICc) criterion was used for the inference of model quality (Akaike, 1974). Influence of a variable

by its addition or dropping from the full model was judged by change in AIC ( $\Delta$ AIC). The model is described as follows:

$$\text{Comet Assay parameter} = \text{Radioactivity} + \text{Sampling Period} + \text{Sex} + \text{Age Class} + \text{Sex} \times \text{Radioactivity} + \text{Age Class} \times \text{Radioactivity}$$

## RESULTS

### *Body burden and rates of environmental exposure*

Magnitude of the body burden of cesium-137 in live bank voles was strongly predicted by the rates of environmental exposure rate at their trapping locations (Fig. 2.3). Results of a simple linear regression accounting for sampling period, are presented in Tab. 2.7 and results of the least squares comparison of the body burden in voles between sampling periods are presented in Tab. 2.8. Variation in the environmental radioactivity and the internal radiation burden in the trapped voles between major sampling areas is shown on Fig. 2.5

### *Comet Assay*

Our main finding was that both measures of individual and environmental radioactivity were generally nonsignificant predictors of the genetic damage in somatic cells of the wild bank voles, *Myodes glareolus*. Namely, in the mixed-effects analyses (Table 2.2; Table 2.3) we found no significant association of the DNA damage levels in blood cells with the body burden of cesium-137 (Table 2.2;  $F(56;248)=0.05$ ;  $P<0.828$ ) or

the environmental exposure rate (Table 2.3;  $F(59;294)=0.1$ ;  $P<0.7499$ ). We observed significant variability in the extent of the comet DNA fragmentation between trapping periods (Table 2.1; Fig. 2.6). Blood samples obtained in the Fall sampling period had lower levels of DNA damage than samples from both Spring periods (Table 2.1;  $F(1;417)=11.47$ ;  $P<0.001$ ), whereas the levels of genetic damage did not vary between the two Spring sampling periods (Table 2.1;  $F(1;417)=0.02$   $P<0.902$ ). The slopes of the “DNA damage to body burden” relationship differed between seasons (“Spring 2014”:  $b_{log} = -0.26$ ,  $t(68) = -2.11$ ,  $P<0.039$ ; “Fall 2014”:  $b_{log} = 0.11$ ,  $t(285) = 1.92$ ,  $P<0.056$ ; “Spring 2015”:  $b_{log} = 0.17$ ,  $t(158) = 1.54$ ,  $P<0.126$ ), however the overall interaction term “Burden×Period” in the full model for DNA damage was not significant ( $F(58;246)=1.45$ ;  $P<0.237$ ), and was excluded from the model. In addition, some samples from the “Fall 2014” sampling period that were mailed overseas showed unusually high levels of damage with large inter-sample variation (Fig. 2.4), possibly due to longer exposure to ambient temperatures, and were excluded from the analysis.

Overall, female voles demonstrated levels of DNA damage, that were marginally smaller than males (Fig. 2.7; “Sex”:  $F(1;417)=3.49$ ;  $P<0.062$ ). When fitted with individual burden as a damage predictor, the significance of this variable in the model increased (Table 2a). Statistically significant effect of the interaction term Sex×Burden (“Sex”:  $F(56;248)=6.71$ ;  $P<0.0101$ ; interaction “Sex×Burden”:  $F(56;248)=5.26$ ;  $P<0.0227$ ) was explained by the difference between non-significant decrease of DNA damage in male voles and non-significant increase of female voles as associated with the increase of radioactivity (Figure 2.7). Weaker associations were found when the exposure rate was used as the radioactivity dose estimate (Fig 2.7; “Sex”:  $F(59;294)=0.60$ ;  $P<0.4404$ ;

interaction “Sex×Exposure”:  $F(59;294)=1.97$ ;  $P<0.1618$ ). The AICc significance test for the interaction terms suggested that the addition of “Sex×Burden” had a positive effect on model predictions ( $\Delta AIC>2$ ), whereas “Sex×Exposure” did not significantly improve the model (Table 2.2; 2.3).

Adopting a previous methodology for age determination using head width (Kallio, et al., 2014), each animal was assigned to an age class (as “adult, older than 10 months”, “young adult” and “juvenile, younger than 2 months”). When the body burden of cesium was used as co-variate, we found a significantly positive Damage~Age association with a marginally significant Age×Burden interaction suggesting lesser DNA damage in the adult group compared to juveniles under elevated radiation conditions (Table 2.2). This pattern was not observed when environmental radiation was used as a proxy for radiation dose (Table 2.3). Overall, we found that the interaction terms Age×Burden and Age×Exposure were not improving either DNA damage or cell density predictions.

Repeatability analysis revealed a high consistency of comet profiles among experimental trials, regardless of the trapping period ( $R^2 = 0.804$ , Fig. 2.9). Examples of intra-individual comet distributions are shown in Table 2.6. Less than 7% of the total sample pool had more than 10% damage difference in replicas and were excluded from the analysis. The exclusion of samples was not associated with either age (Wald  $\chi^2 = 0.01$ ;  $P<0.976$ ), sex (Wald  $\chi^2 = 0.77$ ;  $P<0.381$ ) or trapping area (Wald  $\chi^2 = 0.55$ ;  $P<0.457$ ). Repeated measurements of 30 randomly selected samples with known levels of damage were performed after 1 year of deep-frozen storage and showed a relatively high degree of congruency ( $N=30$ ,  $R^2 = 0.46$ ,  $P<0.011$ , Fig. 2.10).

### *Cell Density*

On average, approximately 270 (range 100 ~ 550) comet images per animal were initially captured and used for analysis. Based on our estimates each slide replicate contained 1  $\mu\text{l}$  of whole blood, and thus 2  $\mu\text{l}$  of blood per individual were used in each experimental setting. On average, the scanning microscope normally captured around seven nucleated cells for every 5  $\text{mm}^2$  of slide area (roughly 1.4  $\text{nucl}/\text{mm}^2$ ; range 0.19 ~ 7.76  $\text{nucl}/\text{mm}^2$ ). Finally, this would result in a concentration of 100-7000 cells with the average being around 1000 cells per 1  $\mu\text{l}$  of whole blood or 10  $\mu\text{l}$  of blood-*RNAProtect*<sup>TM</sup> solution. Samples that resulted in less than 100 total captured nucleoids were excluded from the analysis (13% of the total vole number). The exclusion of samples containing an insufficient number of comets was not explained by sex (Wald  $\chi^2 = 0.43$ ;  $P < 0.512$ ) or age (Wald  $\chi^2 = 1.00$ ;  $P < 0.317$ ). However, we found that samples from CN (contaminated area) were excluded significantly more often than samples from either control regions. (Wald  $\chi^2 = 12.11$ ;  $P < 0.001$ ).

The results of the full model analysis suggested non-significant relationship between cell density and radioactivity estimates; whenever the relative activity of cesium in the body (Table 2.4;  $F(56; 246) = 0.97$ ;  $P < 0.327$ ), or environmental exposure (Table 2.5;  $F(59; 292) = 0.75$ ;  $P < 0.389$ ) was used as a co-variate. Likewise, we found the DNA damage to be the least significant covariate in both models (Fig. 2.11; Table 2.4; 2.5).

However, the samples from the fall sampling period contained a significantly higher number of cells than in both spring periods (Fig 2.11; 2.13; Table 2.1; 2.4; 2.5;  $F(1; 365) = 4.59$ ;  $P < 0.0332$ ). We found no significant interaction of the sampling period with

any other model parameters, and therefore these interaction terms were excluded from further analyses.

Female voles tended to have slightly higher numbers of white blood cells, but the statistical significance was not consistent between the two radiation models (Fig. 2.12; Table 2.1; 2.4, 2.5). The significant effect of interaction Sex×Radioactivity, when predicting cell density, was explained by a difference in the direction of slopes of the association for each sex (Fig 2.12). AICc criterion analysis suggested that accounting for this interaction significantly improved the models, therefore the variable was included in the analysis ( $\Delta AIC > 2$ , Table 2.4).

Inclusion of age as a co-factor revealed no association with relative density of comets, as well as no significant interaction effect of age with radioactivity in the two models (Table 2.4; 2.5). We tested other possible interactions e.g. Age×DNA damage, but neither of them significantly ( $P < 0.05$ ;  $\Delta AIC > 2$ ) improved the model.

#### *Data availability*

Raw data on the DNA damage in the white blood cells from the natural populations of bank voles from the CEZ will become publicly available at the DataDryad.org portal upon publication of the manuscript.

## DISCUSSION

The objectives of this study were to test whether DNA damage in the wild bank voles that inhabit the Chernobyl Exclusion zone increased in response to radioactive



contamination. Previous studies suggested that the *Myodes* populations in the highly radioactive areas of the CEZ suffered a significant drop in the population size due to contamination during the first years after the reactor meltdown (reviewed in Yablokov *et al.* 2009), but consequently were not found to exhibit significant signs of radiation effects decades after the accident (e.g. Matson, *et al.*, 2000). Given such a long history of contamination, we also did not expect any apparently toxic effects associated with the adverse radiation conditions, yet we predicted that rodents with the highest radionuclide burden would show some signs of physically damaged DNA. This was not observed for the blood samples of this murid species in our case. In fact, our findings demonstrate that radiation did not predict a significant part of the variation in the genetic damage across the collected samples. These results could be underestimating the effect of radiation, because, for example, a significant dynamic in body burden was found between sampling periods with an unexpectedly elevated body burden of  $^{137}\text{Cs}$  in 2015 (Table 2.1). Moreover, some of the voles' samples from the most contaminated areas of the CEZ were excluded from the model, because they lacked a reliable cell concentration needed for the analysis (conversely, the cell count was not correlated with radiation in a separate model as well). Also, when roughly corrected for trapping success between different locations at the CEZ, we found that the probability of vole capture in the most contaminated locations in CN was slightly, albeit not significantly, lower (Fig 2.10) than in relatively non-contaminated control areas of CSW and CSE. However, the question of population density requires a more rigorous and focused study. In general, we speculate that the voles may be undergoing selection against individuals with signatures of genotoxic events in DNA. Our results support the suggestion that in some species the mutation rate due to genotoxic activity of

IR is less elevated in the strongly contaminated ecosystems ( $>1$  cGy  $d^{-1}$  of the total absorbed dose, an equivalent of the annual human occupation dose received in one day) than could be predicted by the relative dose-effect curve reported in medical studies of atomic bomb survivors (NRC, 2006, review in O'Connor, 2017). The reasons behind the variability in the genetic response to the IR should be a high priority for further research.

Next, we tested for a difference in the genetic damage between male and female voles. Higher genetic damage in one sex would indicate decreased DNA repair processes (Trecziak, *et al.* 2008). For example, in humans, it was shown that males may express higher levels of single-strand breaks and alkali-labile sites in DNA due to various damaging agents (Hofer, *et al.* 2006; Slyskova, *et al.* 2011). In our case, we demonstrated that sex was a significant factor in the models that predicted genetic damage from the comet assay. Our results are consistent with previously published data, suggesting that males exhibit higher levels of DNA damage (e.g. Bajpayee, *et al.*, 2002; Hofer, *et al.*, 2006). The physiological mechanisms driving this variation remain unknown. One of the proposed explanations appeals to the age-dependent decline in the rate of nucleotide excision repair (NER), which is supposedly different between the two sexes. (Wei, *et al.*, 1993; Uppstad, *et al.*, 2011). Another explanation refers to the activity of estrogen in females, which triggers the biosynthesis of antioxidant enzymes (Slyskova, *et al.* 2011). We note that studies that systematically test sex-specific response to LDIR are very rare in the field of radio-toxicology in general (Strahlenschutzkommission, 2010). Sometimes, low sample sizes prevent researchers from including sex as a cofactor, or the effect may turn out to be statistically unimportant and thus may not receive enough attention for theoretical review.

We call for more research that would emphasize intersexual differences in the effects of LDIR (e.g. Guerquin, *et al.* 2008).

At the time of preparation of this manuscript, no studies reported the use of the automated comet assay technique to approximate an individual cell count. We believe that because the blood samples were collected, stored and processed for the assay in a consistent and standardized manner, the density of comet objects and nucleoids on a slide should be proportional to the cell concentration in the original blood aliquot. In turn, variation in general white blood cell count can serve as an important proxy for immunological status of an organism. (Beldomenico, *et al.*, 2008). Increases in the density of the white blood cells would have indicated a strong immune response, and a drop in the cell count would indicate radiotoxic effects that could have occurred in cell progenitors and may have suppressed their proliferation. Therefore, taken together with DNA damage estimates, the cell counts (and ratios among cell types) may provide insight about genetic damage in the hematopoietic system of an animal. However, experimental studies would be needed to validate this approach. Some radio-immunological research has been conducted on murine species, but the general patterns of immune response remain largely unexplored (Synhaeve, *et al.* 2016). Based on our analyses, sex and sampling period (fall or spring) were more important for predicting cell density than any of the radiation dose estimates. Although there is little information about relative concentration of blood lymphocytes specifically in the wild bank voles *M. glareolus*, the seasonal variation in WBC counts observed in this study was consistent with the study of the immunological status of the wild murines (Beldomenico, *et al.* 2008). It should be noted, however, that our calculations indicated that the re-estimated aliquots contained significantly less PBMCs than expected for the wild

vole. Furthermore, we did not detect any significant sign of cellular damage associated with the blood sampling or preservation.

Finally, we tested for the effect of aging on the genetic damage and immune status of the voles. We expected that older individuals would have decreased DNA repair activity and thus exhibit higher DNA damage. Although we found that some age groups were underrepresented during specific sampling periods (i.e. adult voles >10 m.o. in Fall 2014), overall, age of voles was not significantly associated with DNA damage. In the case of immune status, we expected that older voles would show decrease in cell concentration, however our visualizations suggested, that the concentration of WBCs was non-significantly increasing with age (Fig. 2.13).

#### *The comet assay: taking advantage of limitations*

Single cell gel electrophoresis, (i.e. the comet assay), has become a frequently used tool for *in vivo* and *in vitro* research in many fields of animal toxicology including radiobiology (Olive & Banáth, 2006; Olive, 2009). Various modifications of the method have revealed complex patterns of genotoxicity for a vast spectrum of DNA-damaging agents. Multiple sequential assays using samples from live tissue are used to create a series of “snapshots” that allow inferences of the recovery trajectory after damage (Azqueta, *et al.*, 2014). The method is favored for its applicability for practically all kinds of tissues. However, cell suspensions, i.e. blood (hemolymph), sperm or *in vitro* lymphoid culture systems are preferred for their convenience and reliable approximation of the health status of a subject (Collins, *et al.*, 1997, Collins, 2004, Baumgartner, *et al.*, 2009).

It is assumed that the subsequent alkaline treatment helps detect single- and double-stranded breaks and converges sites with missing bases (AP-sites) into double-stranded breaks (Olive & Banáth, 2006). Surprisingly, in our studies the PBMC of the wild bank voles appeared to be resistant to the standard CA protocol as was inferred from the undetected migration of the nucleoid DNA as we varied the lysis and alkaline buffer incubation time of the samples each for up to 50 hours. To overcome the challenge of non-responsive nucleoids, we followed recommendations to revise the classic protocol by Singh and included an incubation step with a non-specific proteolytic enzyme – proteinase K (PK). The only modification was that we applied the PK incubation step before the salt lysis instead of after – this way we did not expose the already solidified gels to 37°C which could potentially result in gel loss. Originally, the authors of this CA variant assumed that the Proteinase K allows dissociation of DNA-protein crosslinks. (Su & Song, 2008; Valencia, *et al.* 2011). Currently in the comet assay literature this method usually serves as a stand-alone lysis treatment. In our case, the combined treatment provided notably better relaxation of the nuclei under four times lower concentration of the enzyme than in the other studies (~10 µg/ml in the present work versus 40 µg/ml in the study by Valencia *et al.* 2011). We concluded that migration of DNA fragments, which were observed only with the included proteolytic step, together with no relaxation of the nucleoids when the PK treatment was omitted, may suggest significantly more rigid DNA-protein complexes that make up the nuclear scaffold in the white blood cells of wild bank voles. Such strong DNA-protein crosslinks could be contributing to the physical resistance of DNA against damage under oxidative stress, but this hypothesis requires more rigorous assessment.

Unfortunately, tissue sampling during field research is often limited to a single probe per animal per expedition, which is then preserved rather than cultured. Our study demonstrates that preservation of samples induces negligible, if any, extra DNA damage, at least for this species, although inadequate handling and transportation without controlled temperature conditions could significantly affect the performance of the comet assay (Pic., 2.4). Reliability of the method can be determined by the consistency of intra- and inter-experimental replications, which our results certainly demonstrate (Fig. 2.9; 2.10). Yet, we had to account for variation between experimental trials in the statistical models by using the experimental day as a random-effect intercept variable. In this way we demonstrated that a single probe can reliably serve as a snapshot of genotoxic events in an individual when compared with other animals sampled across other expeditions.

The precise chemical composition of RNAprotect™, which was used as a blood preservative in this study, is a trade secret, and therefore we cannot fully speculate on its abilities to absolutely preserve cellular organelles and structures of interest for the comet assay, such as the nucleo-proteomic complexes comprising the chromatin. However, our preliminary analyses of the blood-in-preservative aliquots with Trypan blue showed that the stain did not reveal any noticeable level of apoptosis in the nucleated cells suggesting little to no membrane damage in the preserved samples. Reverse-engineering approaches to mimic “RNAprotect” involve the addition of citrate-based salts and disodium salt of EDTA – which are commonly used to prevent the formation of ice crystals during freezing, as well as to chelate metal ions thus decreasing the activity of enzymes. Qiagen’s RNAprotect™ preservative, according to its supplementary information sheet, also contains tetradecyl-trimethyl-ammonium oxalate, a component also known as Catrimox-

14, which is used for precipitation of nucleic acids (Macfarlane & Dahle,1997). In our preliminary methodological approaches with the comet assay, aliquots of the preserved murine blood samples that were stored in RNAprotect™ at -70°C showed high consistency in comet profiles in bi-annual repeated tests (Fig. 2.10)

There is a consensus in radio-toxicological research that ionizing radiation causes disturbance of molecular biological systems via surges of oxidative radicals, the process known as oxidative damage (Azzam *et al.*, 2011). For *in vitro* research, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a classical simulator of the processes that directly result in DNA damage (single-, double stranded breaks and oxidized bases). For instance, addition of 0.1 mM H<sub>2</sub>O<sub>2</sub> is expected to produce at least as much DNA damage as a dose of 5 Gy of gamma radiation in mammalian cells (Driessens, *et al.* 2009). We also experimented with the H<sub>2</sub>O<sub>2</sub> as a positive control. Unexpectedly, the blood suspension in the preservative seemed to buffer a significant amount of peroxide exceeding most protocol recommendations (e.g. Andreoli, *et al.*, 1999; Driessens *et al.* 2009). As a result, we were not able to establish a calibration curve with defined DNA damage-via-peroxide analog. Such limitations, as the above-described absence of adequate positive controls, which could also have been adjusted for sex and age, overall prevent us from deriving conclusions about absolute levels of the radiation-induced damage. Nevertheless, the consistency in sampling and handling, as well as the repeatable patterns of the genetic damage, confirmed in our analyses (Fig. 2.9; 2.10; Table 2.6), allow us to compare the samples within and across different sampling periods both in terms of the DNA damage and the cell density.

In most research papers, the profile of a migrated (tail) DNA of a nucleoid (comet) sampled is referred to as a measure of “DNA damage”. We think, this needs a bit more

clarification since the actual DNA damage caused by extraneous agents should not be confused with the pattern of fragmentation of DNA, as seen after electrophoresis of nucleoids in the comet assay. Although the positive association between activity of various genotoxic agents and migration of comet DNA has been established, the picture of a “damaged” comet by itself may not specifically tell what exact type of “damage” the formerly living cell had previously experienced. The author’s personal PBMC’s which were sampled and processed in a similar fashion (retroorbital puncture was, however, compromised), and were used as a control for electrophoresis quality, showed on average 30% of “DNA damage” even though he was not subject to DNA damaging agents. Many research studies that used the comet assay report a large within-group variation for most comet parameters (Garm, *et al.* 2013). In our experiments, the patterns of fresh and long-term preserved human PBMC were congruent through multiple runs over several years of experimentation, while in parallel runs the samples of murine species showed variability in comet shapes (Table 2.6). We believe that DNA damage caused by genotoxic event does positively contribute to the comet fragmentation levels. However the stand-alone average estimate of the comet-induced DNA damage provides little to no useful information beyond the context of a damaging factor.

We believe that results of our studies will have important implications for future research in radioecology. We have created a large library of fixed samples of nuclear DNA, which can be preserved in its agarose-sealed state for decades without degradation. This library may serve as a convenient genetic snapshot of the *Myodes* population of the Chernobyl Exclusion Zone. In the future, methods can be developed (e.g. involving



fluorescent DNA probes), that would genotype the samples as well as identify qualitative molecular patterns of the radiation damage, e.g. levels of oxidized nucleotides.

### *Murine radioecology: the mysterious Myodes*

Historically, various native mice and voles were envisioned as sentinel species for studies of environmental IR. In fact, the lack of the expected mortality in some of wild-caught rodents was first noted in the first radioecological evaluations of the accident at the “Mayak” Plutonium plant (USSR) in 1959 (e.g. Il’enko, 1974). Subsequent assessments of the contamination produced by the so-called East Urals Radioactive Trace (e.g. Grigorkina, *et al.*, 2006; 2007; 2013) attempted to resolve the exceptional radio-resistance of the *Myodes* voles as compared to other closely-related murids. Parallel experiments on naïve red-backed voles, *M. gapperi*, from natural populations in Manitoba, Canada, had also demonstrated that acute whole-body gamma irradiation of these murids did not produce significant effects on their longevity (Mihok, *et al.*, 1985). Some studies of wild rodents were also conducted in the northern part of Ukraine (at the time former Ukrainian SSR) before the reactor meltdown at the Chernobyl NPP in 1986 which helped facilitate a baseline population for further studies, when the accident occurred (e.g. Bashlykova, 1999; review in Ryabokon & Goncharova, 2006; Yablokov, 2009).

The results of the earlier studies of these populations, which experienced the Chernobyl fallout, demonstrated patterns of significantly greater genetic effects due to LDIR. Preliminary findings on *Microtus sp.* were published shortly after the Chernobyl accident (Bashlykova *et al.* 1987) demonstrating an increase in the micronuclei rate in the affected voles’ blood cells. Cristaldi, *et al.* (1991), reported that the proportion of

micronucleated polychromatic erythrocytes was significantly positively associated with the content of radioactive cesium in muscle tissue, sampled from the voles, affected by contamination by this radioisotope in South-Eastern Sweden. Goncharova *et al.* (2000) were also studying voles in the Exclusion zone and also reported weakly elevated frequencies of micronuclei and other chromosomal aberrations in sampled *M. glareolus*. Later, they also reported elevated embryonic mortality in bank voles and this was attributed to radioactive contamination (Ryabokon & Goncharova, 2007). Conversely, other studies reported no detectable increase in the micronuclei fraction in the naïve voles and laboratory mice, i.e. those that were introduced to the radioactive environment of the CEZ without pre-radiation history (e.g. Rodgers, *et al.* 2001; 2010). This, perhaps, could not have been expected, given that priming by IR had been shown to reduce the effects of radiation, meaning that naïve irradiation produces the most severe effects (Amundson *et al.* 2003). In a recent study, however, an increase in genomic mitochondrial variability in the wild voles from the Red Forest in the CEZ was reported (Baker, *et al.*, 2017). Albeit, consequences for mitochondrial function remain unknown (Boratyński, *et al.*, 2011). From these studies, it could be generally inferred that we would likely observe much weaker effect of LDIR 30 years after the accident, which seems reasonable, as the effective dose is expected to decrease due to decay of the relatively short-living isotopes dominant in the ecosystem immediately following the accident.

Our studies confirm that bank voles *M. glareolus* in the contaminated areas of the CEZ are still exhibiting significantly higher body burdens of cesium compared to other measured murine species (Chesser *et al.*, 2000; 2001). Because of this, we expected that our trapped animals would carry at least some markers of genetic response to such

radioactivity load. We think that the key to understanding the variation in radio-resistance properties of these mammals may be in their metabolic management of the oxidative stress – according to our meta-analysis murine species tend to show very weak positive antioxidant response in relation to the increased radioactivity (Einor et al, 2016).

In general, the biological effects of environmental contamination in highly mobile animals, should be addressed with respect to precise estimate of their accumulated radiation dose. Unfortunately, our study is significantly limited by not accounting for internal beta-emitting isotopes (such as strontium-90) and by not having measured the soil contamination at the trapping sites – this makes it difficult to establish a reliable dose estimate for an individual rodent (e.g. using dose conversion coefficients as in Ulanovsky & Pröhl, 2014, or via general estimation, e.g. after Garnier-Laplace, *et al.*, 2015). Nonetheless, the results of our empirical research indicate that usage of the other two independent factors of radioactivity show similar patterns of the somatic and genetic response in the blood cells. Future studies should account for soil contamination by radionuclides, and properties of the sampling habitats together with vegetation and climate data for more precise modeling of individual acquired doses.

### *Conclusion*

There are many potentially fruitful directions for research that could further investigate low dose effects on natural populations. According to Timofeev-Resovkiy (1974), any abrupt and unexpected changes in the environment should be reflected in shifts of the genetic composition of a population. If the generation turn-over rate in this population is high, the speed of the allelic shift would also be relatively rapid in response

to the environmental change (Hoffmann & Will, 2008). In the case of a chronic hazard, such as radioactive pollution, the selection for the *most fit* individuals within populations would seem like a reasonable outcome (Ilyenko, 1974; Galvan *et al.*, 2014). Here, the “most fit” could be interpreted as “selected for the survival and reproductive success under highest sustained effective dose”. In this case, adaptation by natural selection would require variation of the phenotypes, which would be differently performing under LDIR conditions, and the heritable genetic basis, which would explain this phenotypic variation. Unfortunately, to date, there have been very few studies investigating adaptive responses in natural populations and most fail to meet the least stringent requirements for demonstration of adaptive evolutionary responses. (Møller & Mousseau, 2016). Current theory predicts that increase in mutation rate would also bring about increased accumulation of selectively neutral and near-neutral mutations. Increase in mutation burden under conditions of declining carrying capacity would likely lead to the extinction of a population (Higgins and Lynch, 2002).

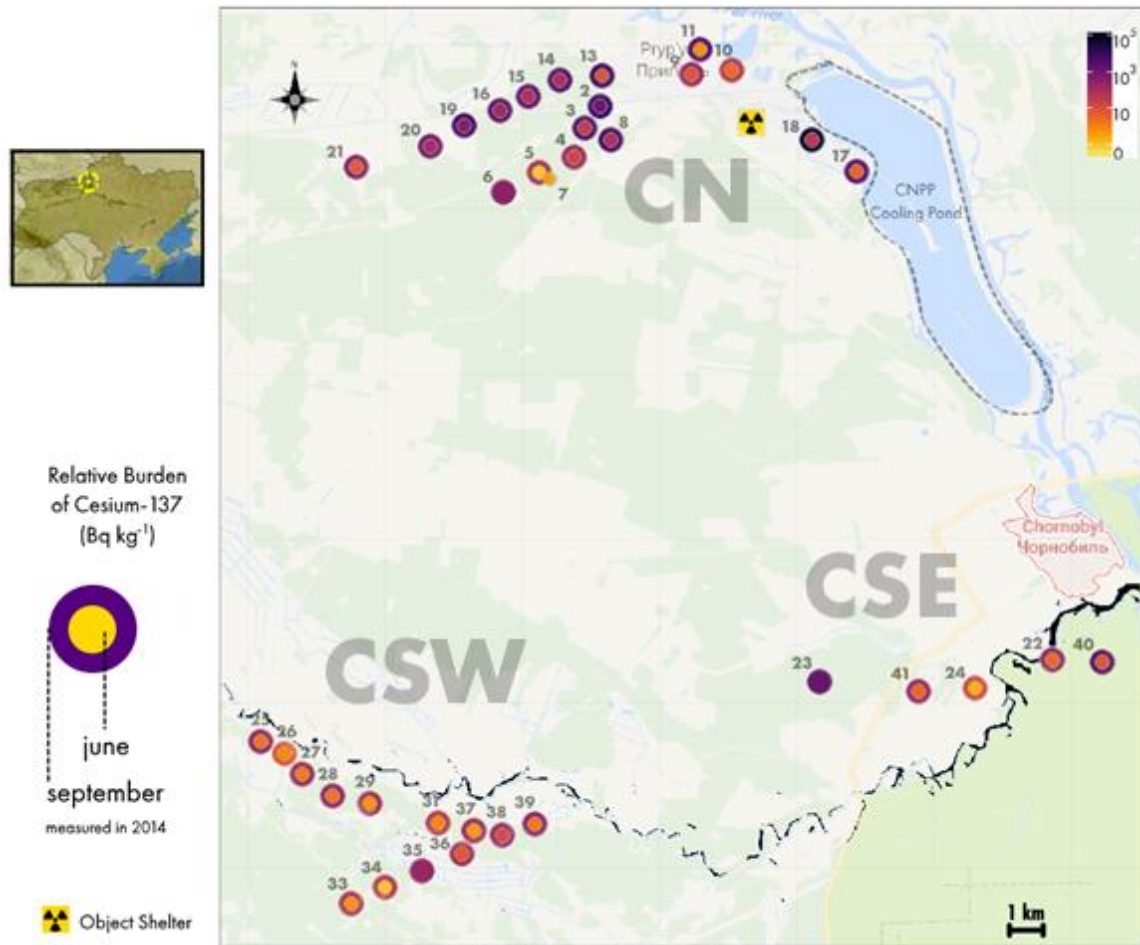
Recent frequent occurrences of severe weather events together with the aging of the currently operating nuclear reactors raise concerns about unregistered release of radionuclides (Mousseau & Møller, 2017) or the possibility of a *force majeure*, e.g. a dangerous natural incident leading to another Fukushima-scale disaster, which has been given at least 50% likelihood of re-occurrence by 2050 (Wheatley, *et al.*, 2016). The problem of the long-term effects of radioactive contamination requires further attention to scientifically assess, prevent and mitigate possible consequences of the radioactive pollution on human health and biota. Meeting these challenges will require complex and

well-managed approach involving rigorous sampling and sophisticated forensic-quality techniques with a significant investment of financial and human resources.

#### ACKNOWLEDGEMENTS

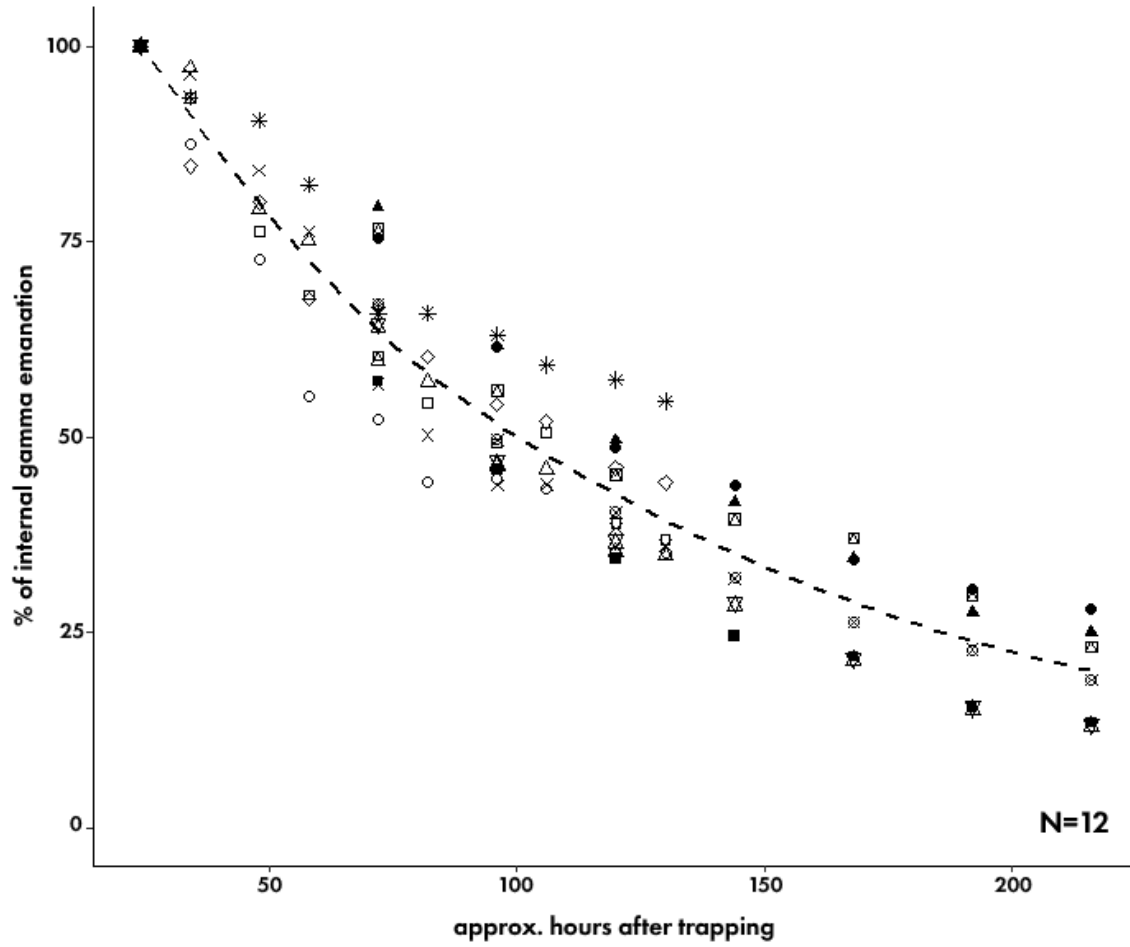
The author would like to thank Dr. Z. Shelest, Dr. A. Bonisoli-Alquati, Dr. M. Wyatt and Dr. A. Waldman for their extremely helpful suggestions for the experimental part of the manuscript.

## FIGURES AND TABLES



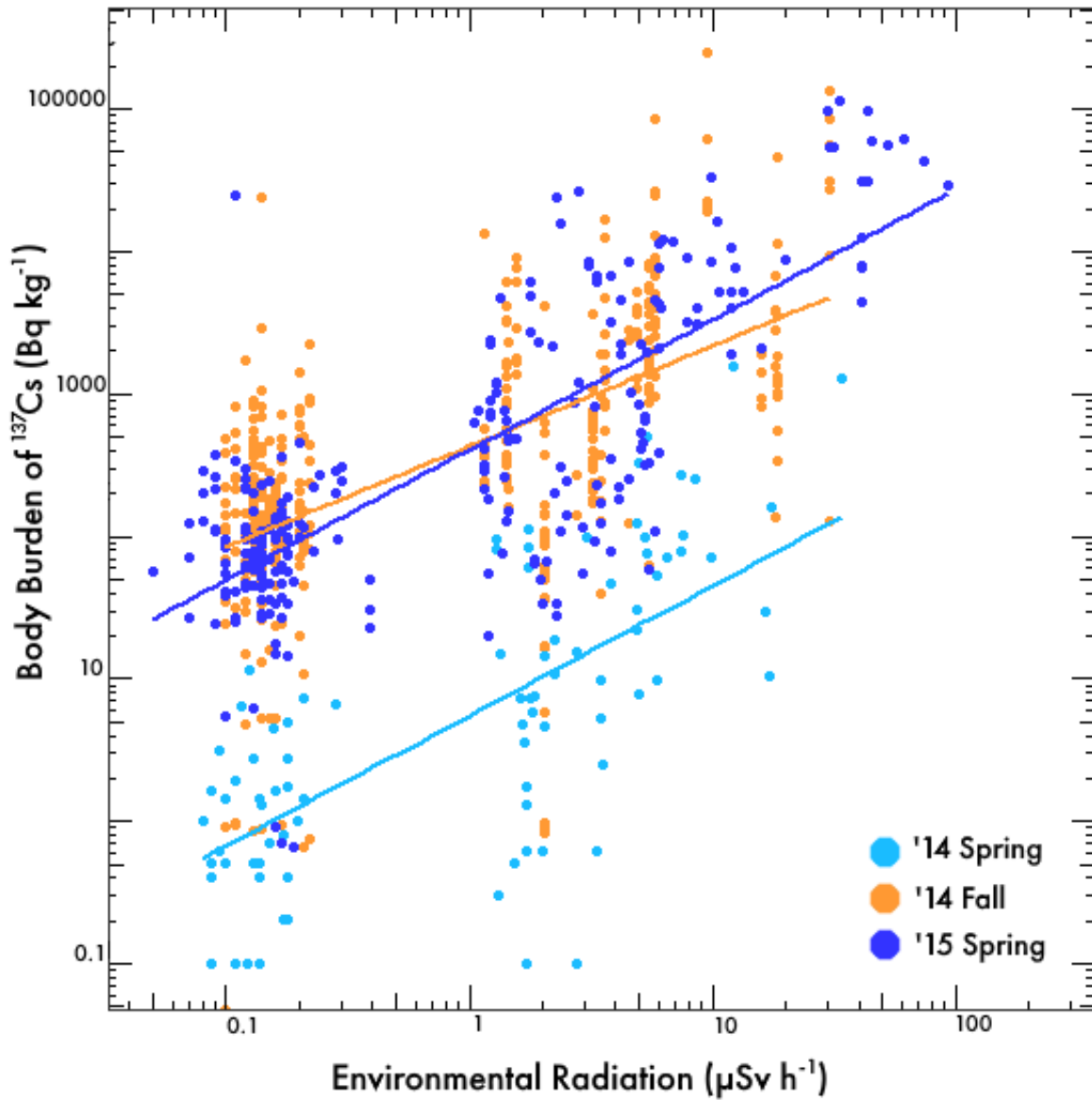
**Figure 2.1** Map of the Chernobyl Exclusion Zone (CEZ) showing the locations of vole trapping sites<sup>9</sup> within the experimental (CN) and control areas (CSE, CSW).

<sup>9</sup> On the map, approximate disposition of trapping sites is shown. The Uzh river is contrasted. Sampling sites are colored in a gradient fashion (R library *viridis*) proportionally to the mean estimates of the cesium-137 body burden found in the bank voles.



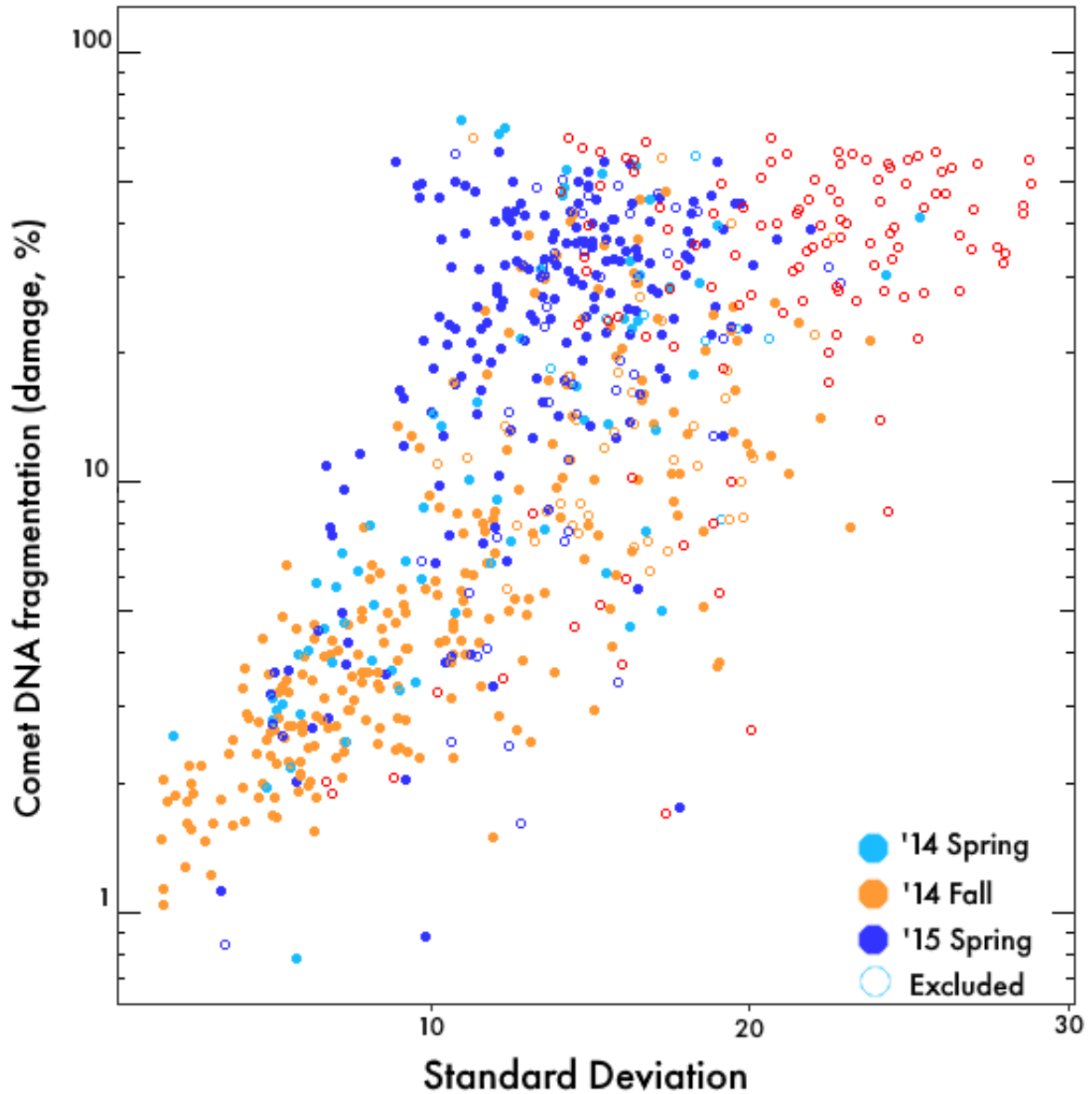
**Figure 2.2** Loss<sup>10</sup> of the radioactivity burden (in %) due to excretion in live bank voles.

<sup>10</sup> Dotted line represents the projected mean produced by the *loess* function in *ggplot2*. Repeated measurements for each animal are depicted in distinct shapes.

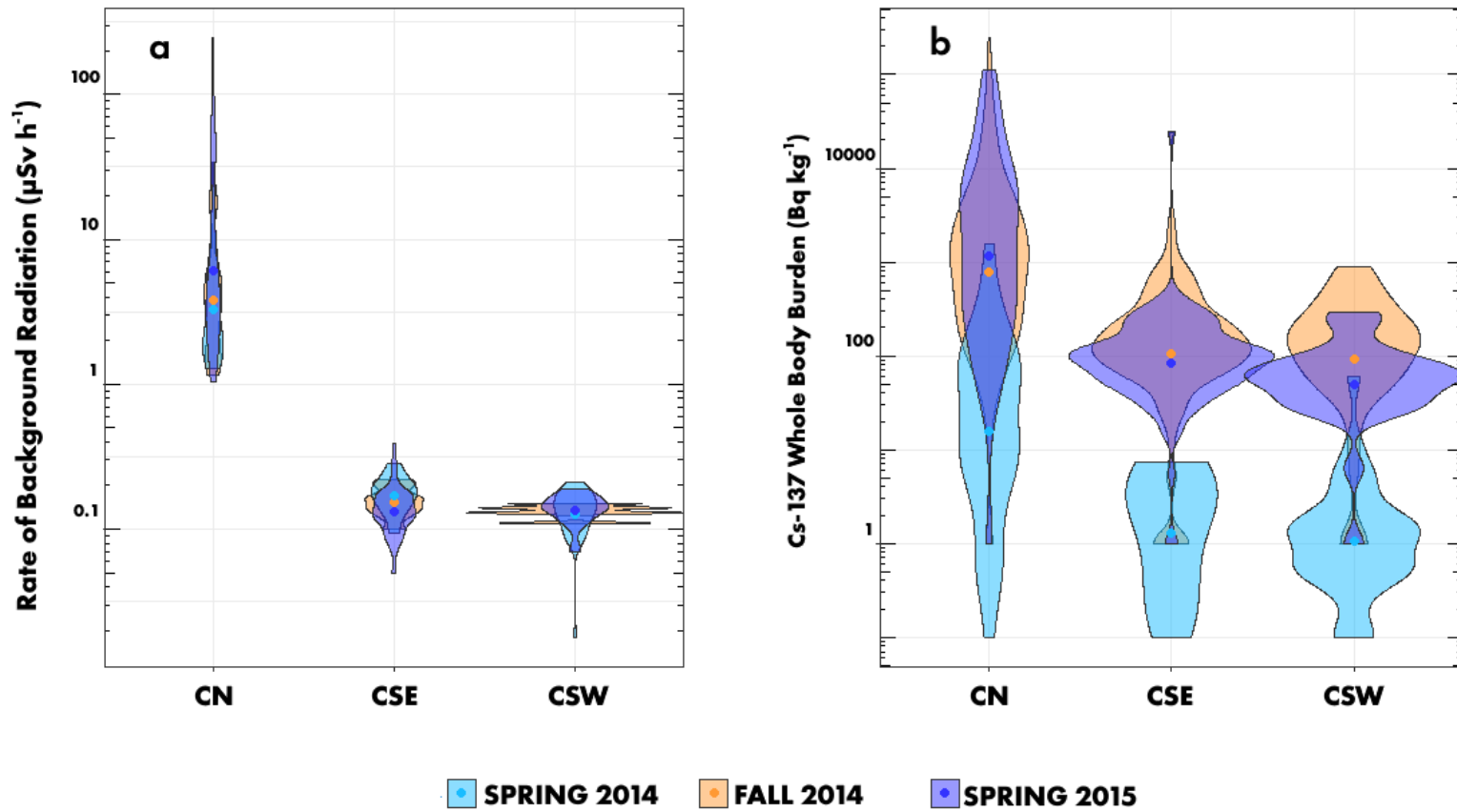


**Figure 2.3** Scatter- and linear plotting of the association of the whole-body burden of cesium-137 ( $\text{Bq kg}^{-1}$ ) in live bank voles with the average rate of environmental  $\gamma$ -radiation ( $\mu\text{Sv h}^{-1}$ ) in their habitat.



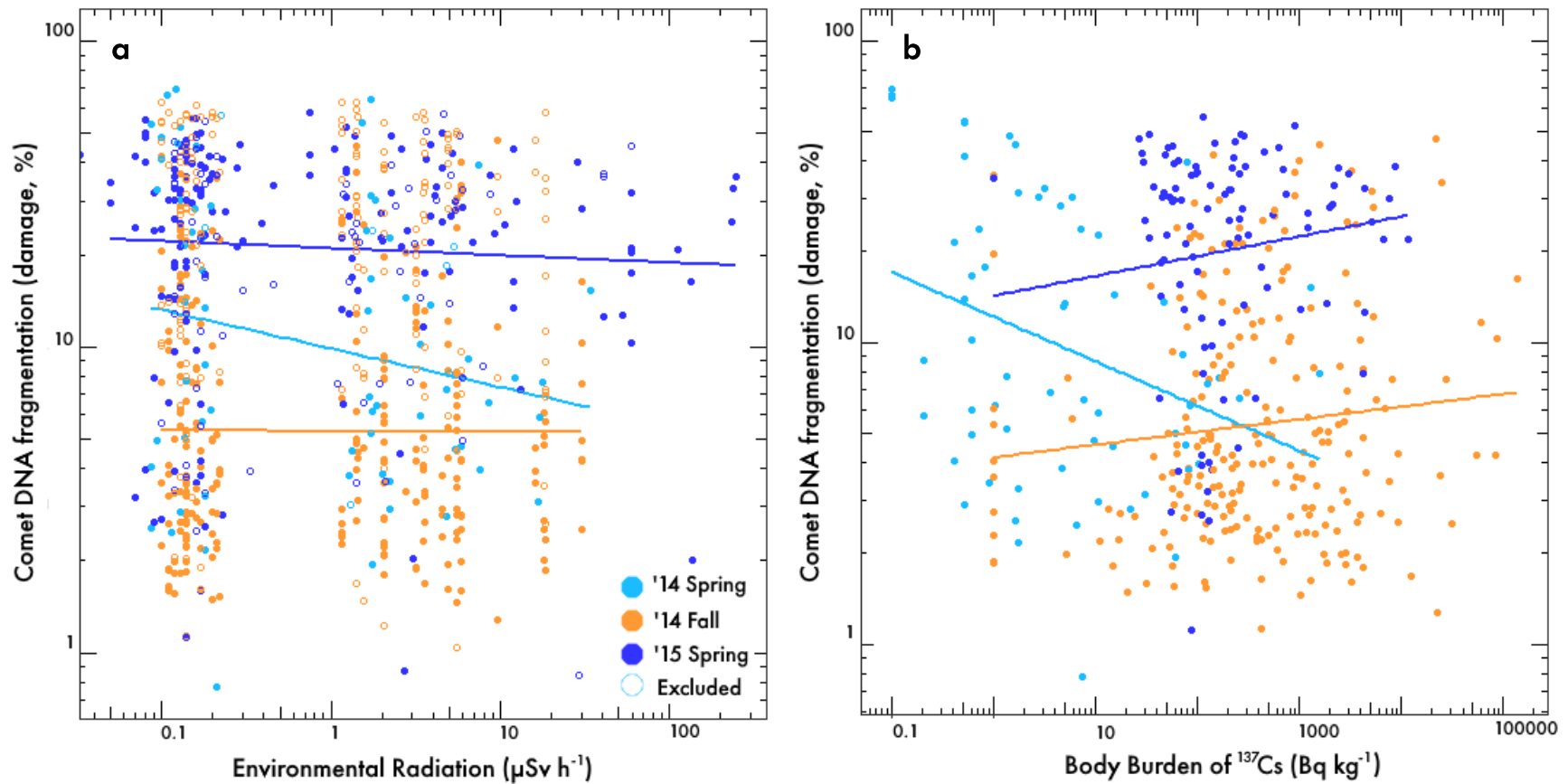


**Figure 2.4** Scatterplot for the comet assay estimates (median % of tail DNA) versus variation of the mean (SD) demonstrating effect of sample mishandling (red, excluded from analysis).

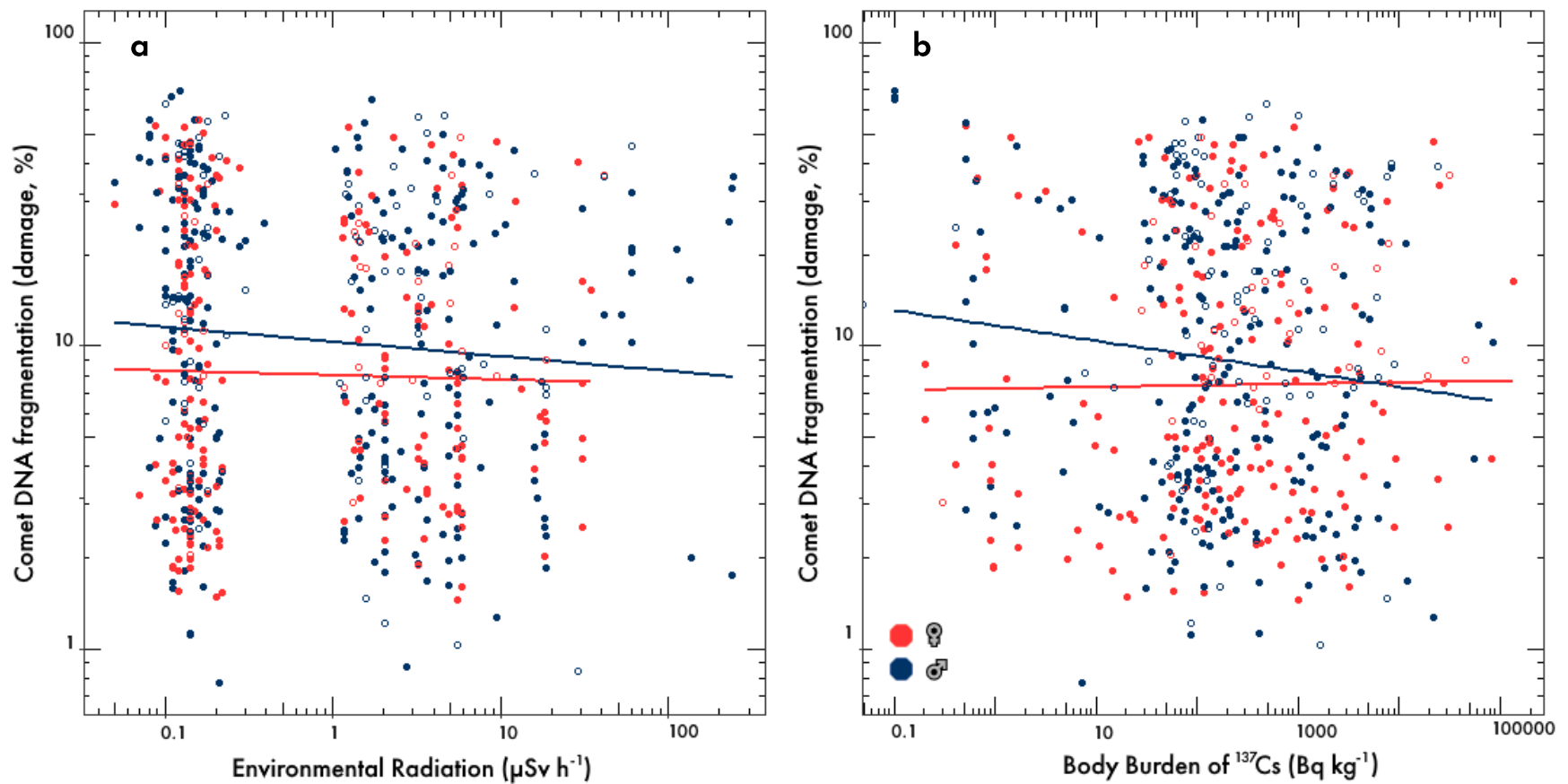


**Figure 2.5** Variation (violin plots) of (a) the rates of environmental radioactivity at trapping locations and (b) the average cesium-137 body burden in live voles grouped by general experimental locations within the CEZ<sup>11</sup>

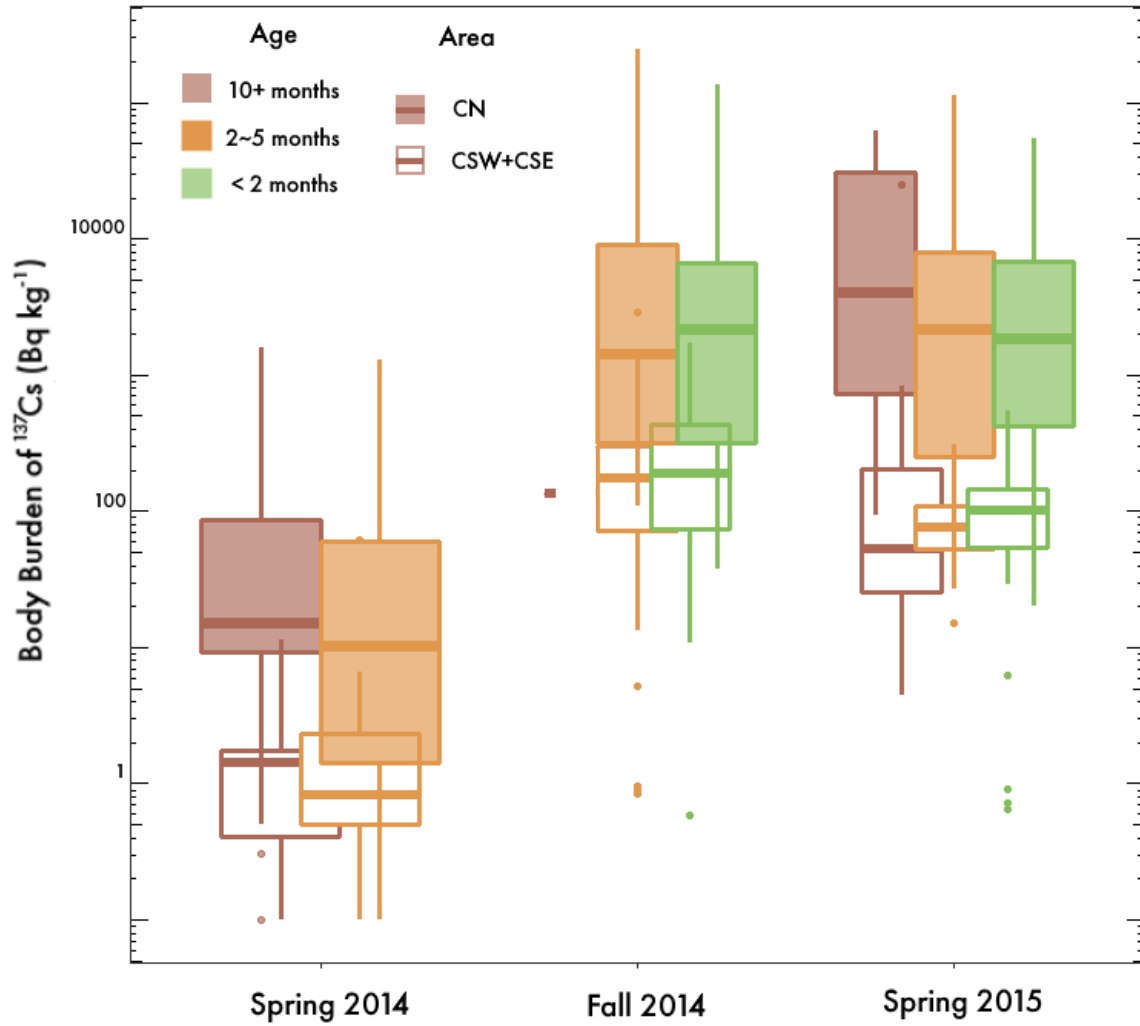
<sup>11</sup> (Contaminated: CN – Chernobyl North; Control: CSW – Chernobyl South-West; CSE – Chernobyl South-East)



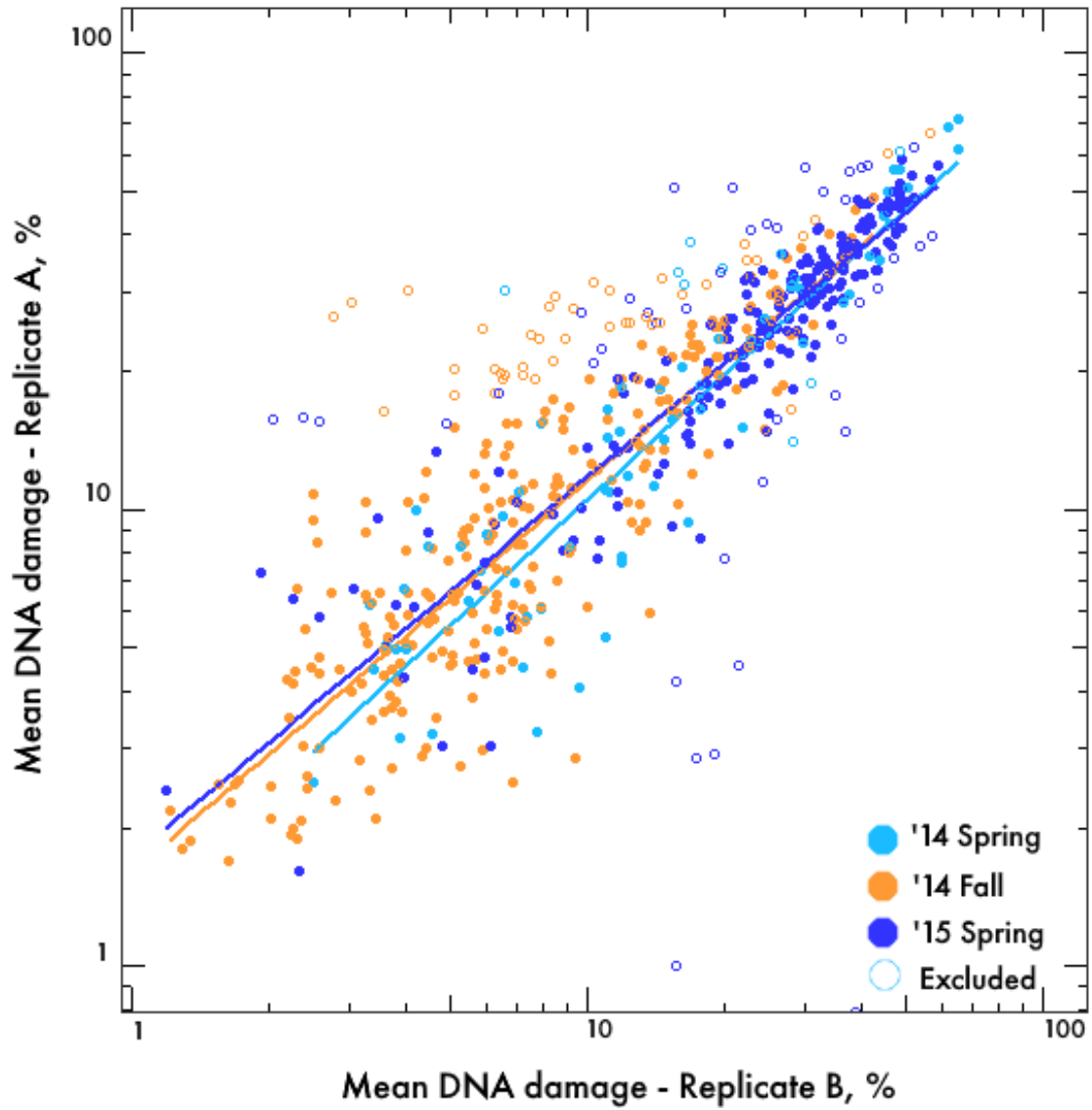
**Figure 2.6** Scatterplots for the levels of DNA damage in preserved white blood cells of the bank voles versus (a) the rate of the environmental exposure in their habitat; and (b) the estimated body burden of cesium-137 in live animals caught in their respective sampling period



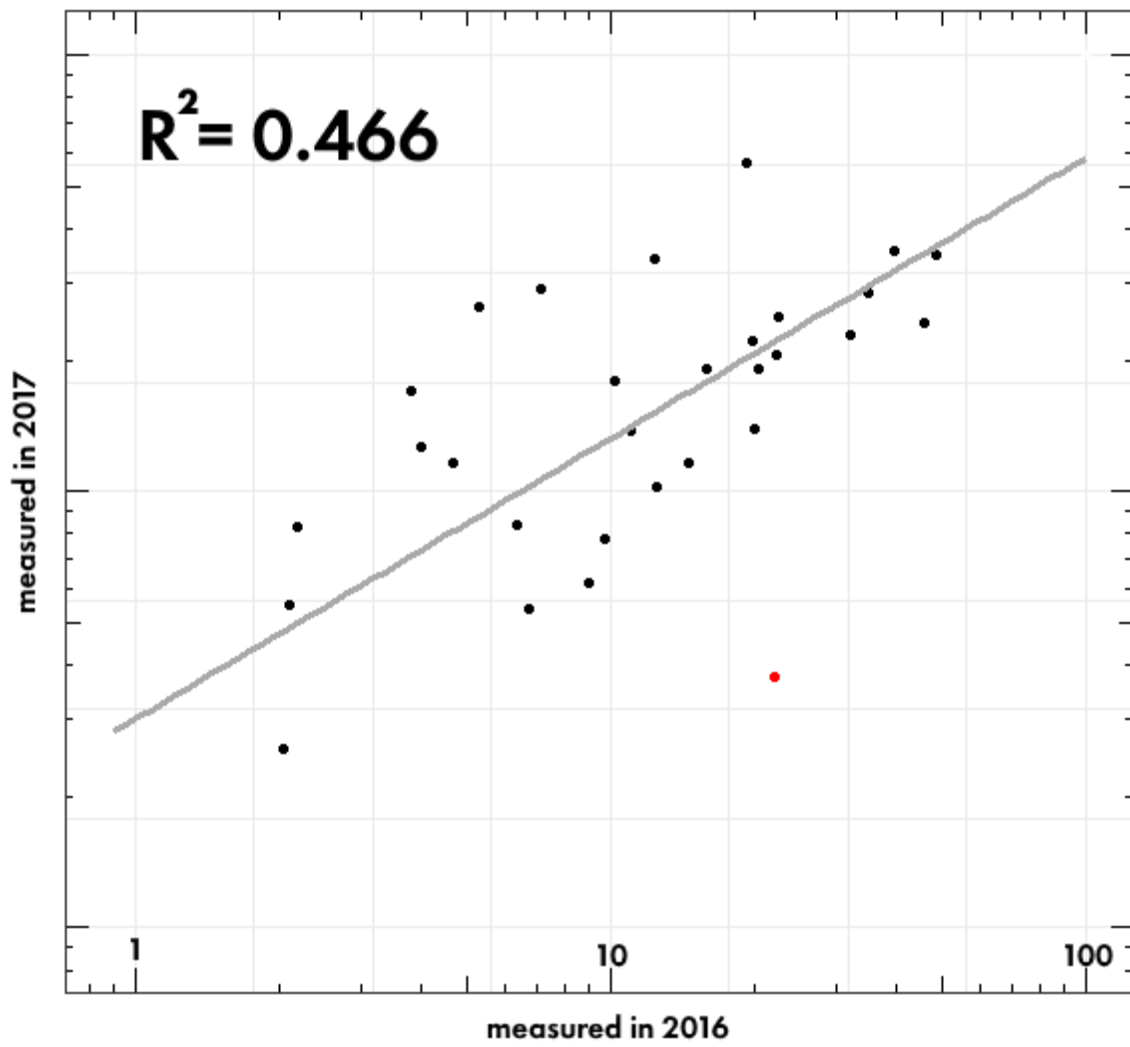
**Figure 2.7** Effect of sex on the association of DNA damage in the preserved WBC from the bank voles and (a) the rate of environmental exposure and (b) whole body burden of cesium-137



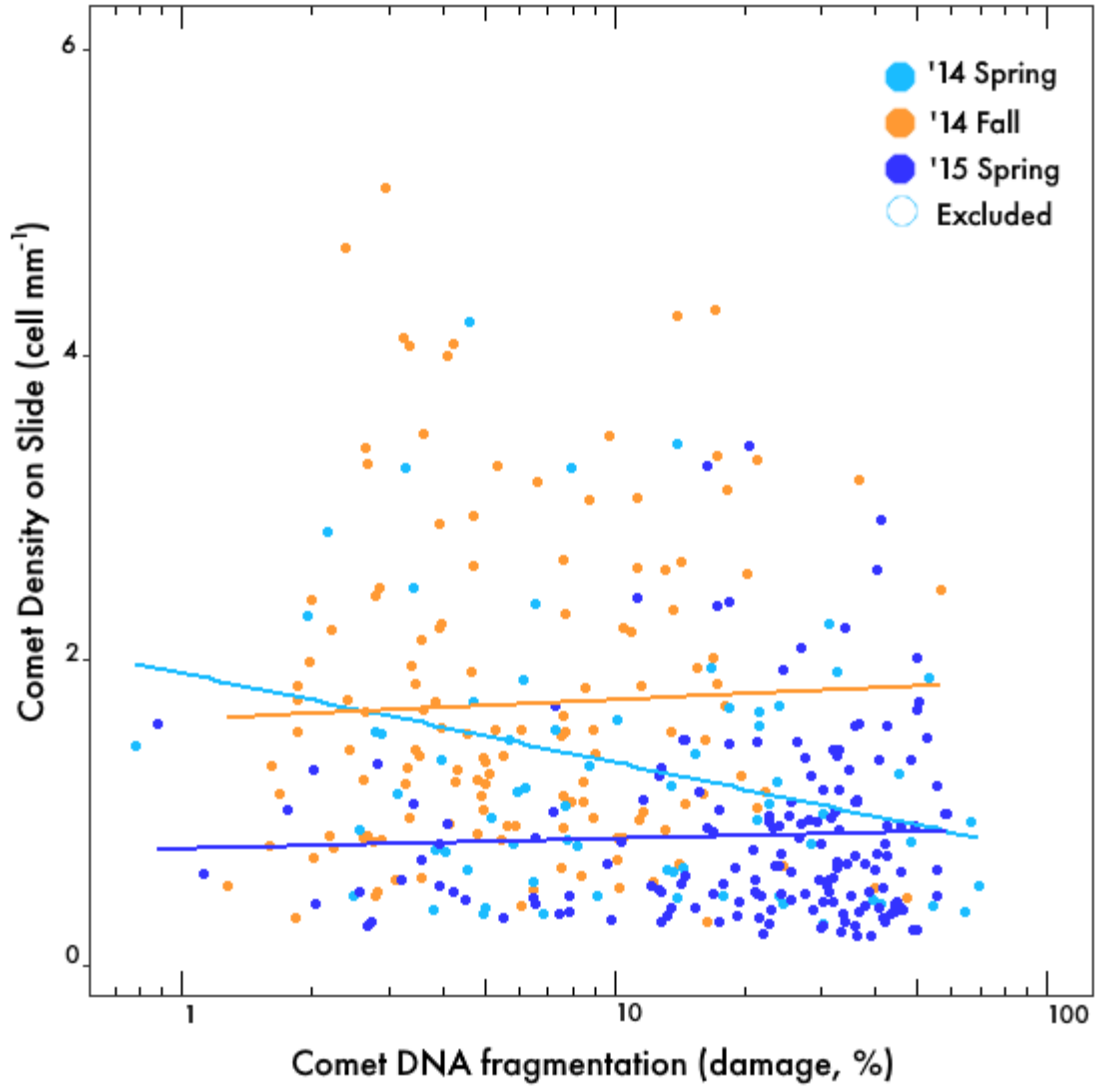
**Figure 2.8** Box-and-whisker plots for the body burden of cesium-137 in sampled voles (grouped by age and sampling period) that compare control and contaminated areas within the CEZ.



**Figure 2.9** Scatterplot for the repeatability analysis (comparison of replicates) of the results of comet assay runs conducted after each sampling period, respectively.

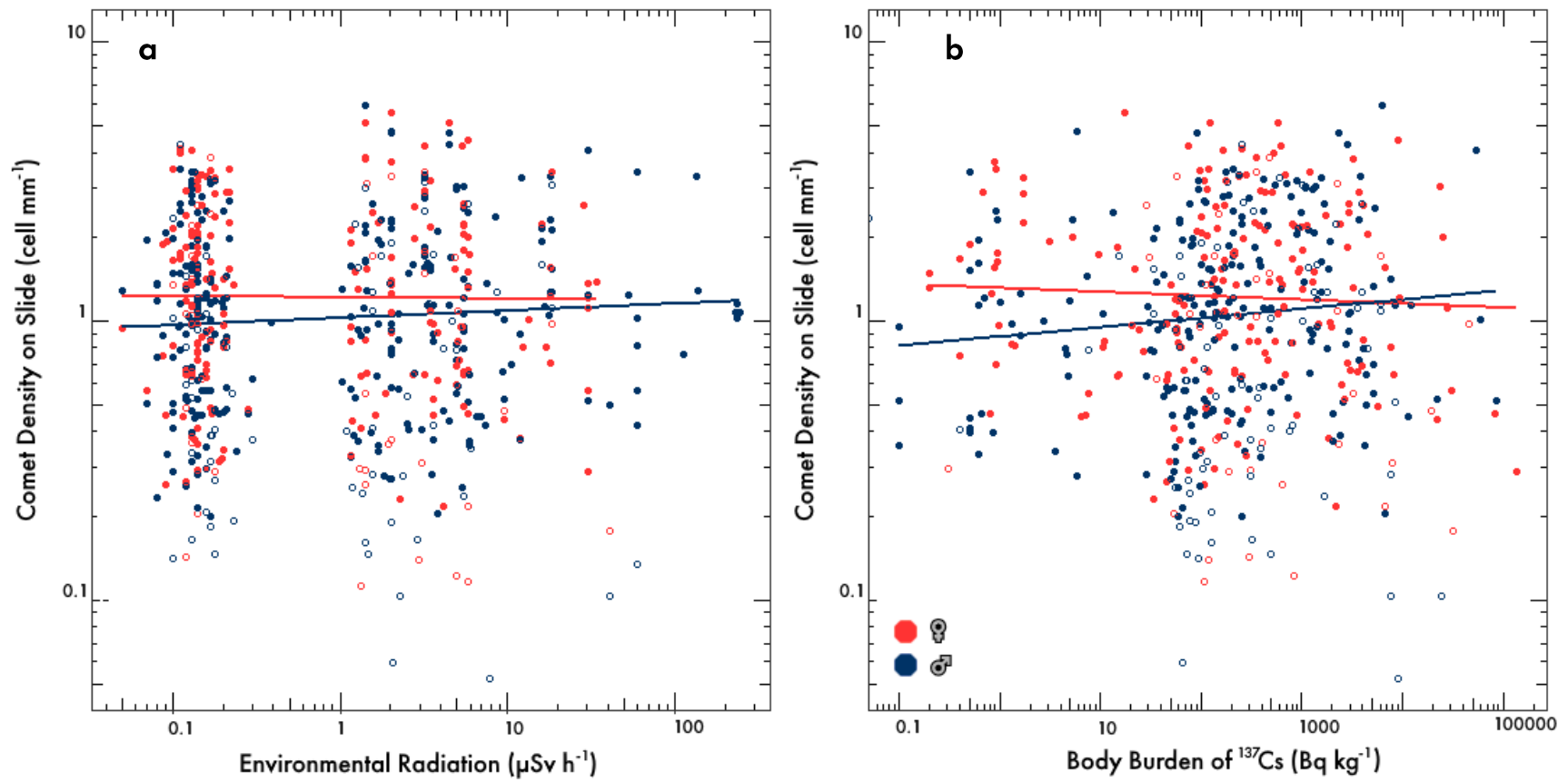


**Figure 2.10** Reproducibility of the comet assay method as shown by re-measuring samples from the Fall 2014 sampling period.

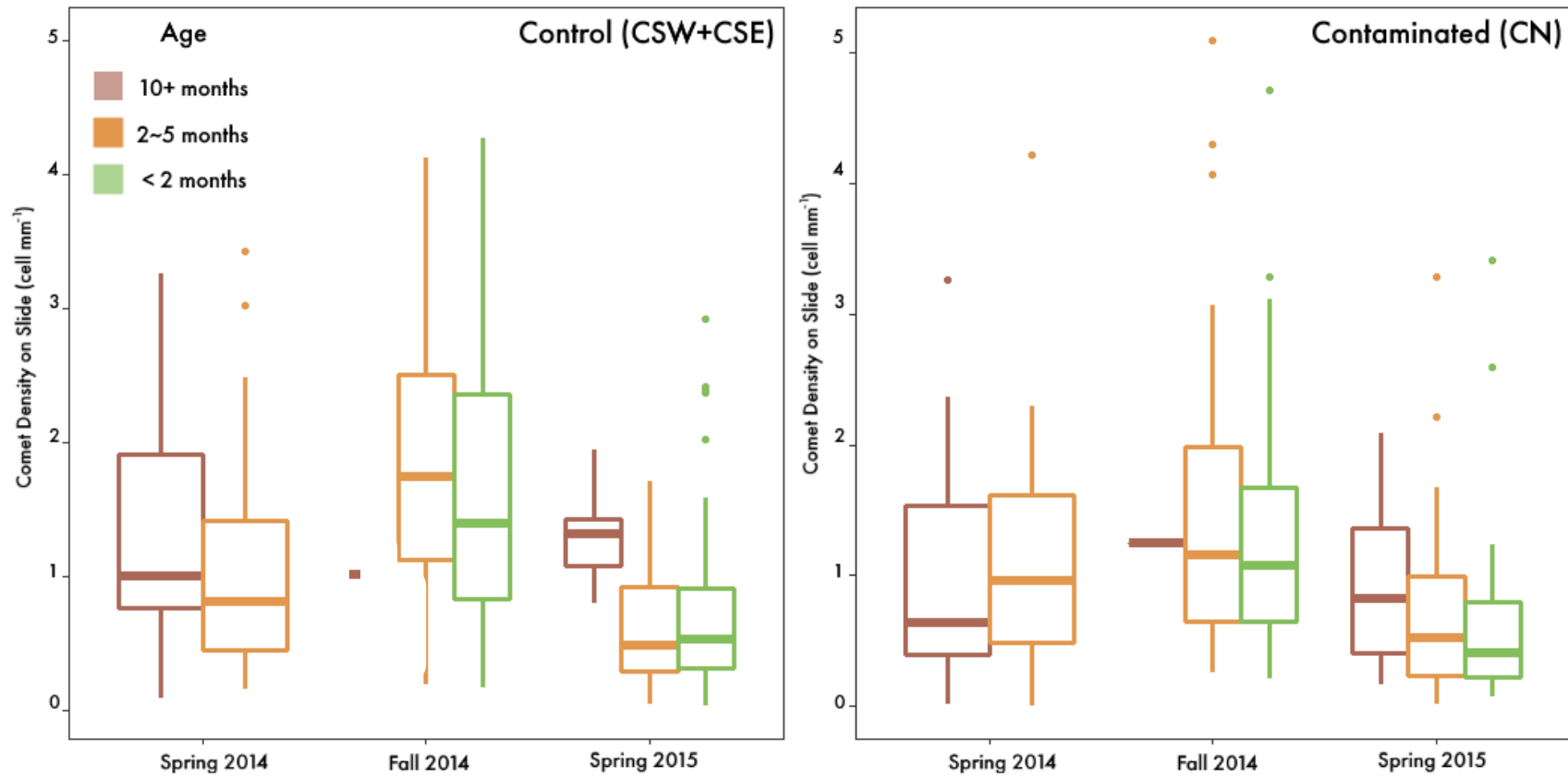


**Figure 2.11** Scatterplot for the association of DNA damage in wild bank voles' WBC and their relative concentration in a blood aliquot





**Figure 2.12** Effect of sex on the association of relative amount of WBC in blood aliquot and: (a) the rate of environmental exposure and (b) the whole-body burden of cesium-137 in live bank voles.



**Figure 2.13** Box-and-whisker plots for the relative amount of WBC in the blood aliquots of the bank voles, grouped by age

**Table 2.1** Means (SE) of the parameters of <sup>137</sup>Cs body burden, genetic damage, and relative cell count in blood sample, provided for the three experimental areas within the CEZ. <sup>12</sup>

Period\Area	Cesium Body Burden (Bq kg <sup>-1</sup> )				Comet DNA damage (%)				Cell Density (mm <sup>-1</sup> )			
	Males		Females		Males		Females		Males		Females	
<b>Spring 2014</b>												
CN	98.11	(46.7)	108.41	(55.37)	17.42	(3.00)	15.38	(2.36)	1.16	(0.15)	1.34	(0.29)
	n=34		n=24		n=25		n=13		n=25		n=13	
CSE	2.29	(1.33)	3.17	(1.31)	24.22	(8.96)	16.27	(8.30)	1.17	(0.37)	1.17	(0.48)
	n=5		n=4		n=5		n=3		n=5		n=3	
CSW	1.42	(0.36)	6.41	(4.28)	27.24	(5.01)	21.49	(5.04)	1.07	(0.19)	1.27	(0.26)
	n=17		n=14		n=16		n=13		n=16		n=13	
<b>Fall 2014</b>												
CN	3627.68	(977.46)	6332.22	(2180.12)	13.35	(1.23)	12.50	(1.12)	1.86	(0.13)	1.89	(0.16)
	n=124		n=136		n=78		n=67		n=78		n=67	
CSE	208.83	(28.13)	485.19	(241.42)	12.39	(2.11)	11.14	(1.32)	1.49	(0.15)	1.90	(0.15)
	n=75		n=100		n=33		n=43		n=33		n=43	
CSW	162.35	(26.74)	227.07	(40.70)	14.84	(1.71)	11.83	(1.95)	1.99	(0.21)	1.97	(0.24)
	n=43		n=36		n=25		n=21		n=25		n=21	
<b>Spring 2015</b>												
CN	8663.47	(1905.94)	6053.50	(2520.10)	28.50	(1.41)	26.90	(2.66)	0.92	(0.07)	0.80	(0.10)
	n=97		n=48		n=74		n=26		n=73		n=26	
CSE	469.31	(367.03)	133.85	(18.32)	25.64	(1.71)	25.30	(2.60)	0.89	(0.08)	0.95	(0.13)
	n=67		n=36		n=54		n=31		n=54		n=31	
CSW	65.01	(12.91)	97.11	(32.43)	34.25	(3.31)	30.52	(5.22)	0.65	(0.14)	0.37	(0.06)
	n=18		n=9		n=14		n=5		n=14		n=5	

<sup>12</sup> (Contaminated: CN – Chernobyl North; Control: CSW – Chernobyl South-West; CSE – Chernobyl South-East)

**Table 2.2** Results of the full GLMM model predicting DNA damage in WBC given the estimates of the whole-body burden of cesium-137.

**DNA damage ~ <sup>137</sup>Cs Body Burden**

**N=305; df = 248; AICc=790.0**

Effect	Categories	$\beta$	(SE)	F	P	$\Delta$ AIC
Burden		-0.0324	(0.0872)	0.05	0.8288	
Period	Spring 2014	<b>-0.7677</b>	<b>(0.3409)</b>	<b>11.07</b>	<b>&lt;0.0001</b>	
	Fall 2014	<b>-1.4539</b>	<b>(0.3103)</b>			
	Spring 2015	0				
Sex	Females ♀	<b>-0.4846</b>	<b>(0.1956)</b>	<b>6.71</b>	<b>0.0101</b>	
	Males ♂	0				
Age	Adults	<b>0.7094</b>	<b>(0.3475)</b>	<b>3.74</b>	<b>0.0250</b>	
	Young adults	0.1176	(0.3022)			
	Juveniles	0				
Burden*Sex	Females ♀	<b>0.1870</b>	<b>(0.0816)</b>	<b>5.26</b>	<b>0.0227</b>	+2.1
	Males ♂	0				
Burden*Age	Adults	<b>-0.3292</b>	<b>(0.1565)</b>	2.42	0.0911	+0.2
	Young adults	-0.0514	(0.1137)			
	Juveniles					

**Table 2.3** Results of the full GLMM model predicting DNA damage in WBC given the rates of environmental exposure to gamma radiation.

**DNA damage ~ Background Exposure Rate**

**N=354, df=294; AICc=926.1**

<b>Effect</b>	<b>Categories</b>	<b><math>\beta</math></b>	<b>(SE)</b>	<b>F</b>	<b>P</b>	<b><math>\Delta</math>AIC</b>
Exposure		-0.0184	(0.0978)	0.10	0.7499	
Period	Spring 2014	-0.5594	(0.3449)	<b>8.05</b>	<b>0.0004</b>	
	Fall 2014	<b>-1.3363</b>	<b>(0.3438)</b>			
	Spring 2015	0				
Sex	Females ♀	-0.0730	(0.0947)	0.60	0.4404	
	Males ♂	0				
Age	Adults	0.0192	(0.1702)	0.06	0.9443	
	Young adults	-0.0254	(0.1053)			
	Juveniles	0				
Exposure*Sex	Females ♀	0.1513	(0.1079)	1.97	0.1618	-0.6
	Males ♂	0				
Exposure*Age	Adults	0.1207	(0.1207)	1.42	0.2445	-1.4
	Young adults	0.1850	(0.1850)			
	Juveniles					

**Table 2.4** Results of the full GLMM model predicting relative WBC count given the estimates of the whole-body burden of cesium-137.

**Cell Density ~ <sup>137</sup>Cs Body Burden**

**N=303; df = 246; AICc=732.6**

Effect	Categories	$\beta$	(SE)	F	P	$\Delta$ AIC
DNA damage		-0.0044	(0.0527)	0.01	0.9340	
Burden		0.0824	(0.0884)	0.97	0.3266	
Period	Spring 2014	0.0824	(0.0884)	<b>10.87</b>	<b>&lt;0.0001</b>	
	Fall 2014	<b>1.3313</b>	<b>(0.3588)</b>			
	Spring 2015	0				
Sex	Females ♀	<b>0.4911</b>	<b>(0.1799)</b>	<b>7.45</b>	<b>0.0068</b>	
	Males ♂	0				
Age	Adults	0.0099	(0.3166)	0.75	0.4726	
	Young adults	0.2242	(0.2729)			
	Juveniles	0				
Burden*Sex	Females ♀	<b>-0.1785</b>	<b>(0.0746)</b>	<b>5.73</b>	<b>0.0175</b>	+2.2
	Males ♂	0				
Burden*Age	Adults	0.2144	(0.1426)	1.72	0.1817	-1.7
	Young adults	-0.0241	(0.1025)			
	Juveniles					

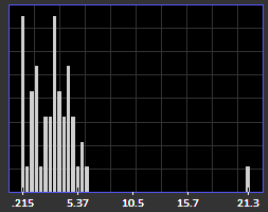
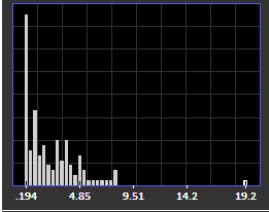
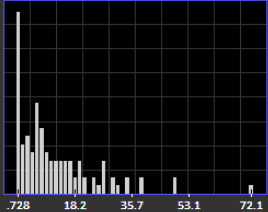
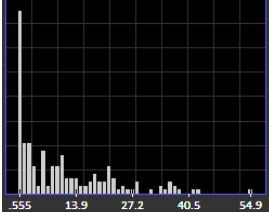
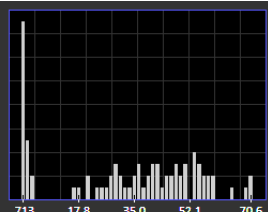
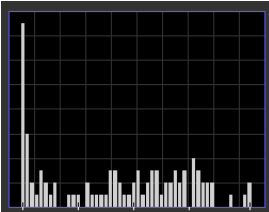
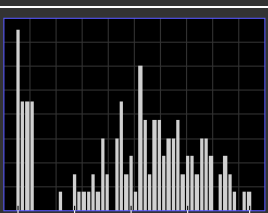
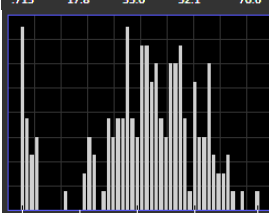
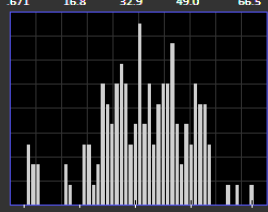
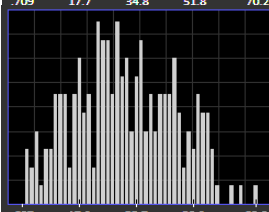
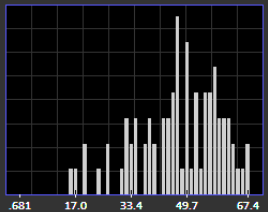
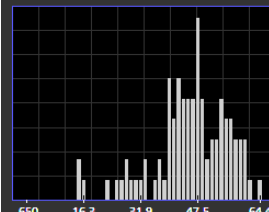
**Table 2.5** Results of the full GLMM model predicting relative WBC count given the rates of environmental exposure to gamma radiation.

**Cell Density ~ Background Exposure Rate**

**N=353, df=292; AICc=816.4**

<b>Effect</b>	<b>Categories</b>	<b><math>\beta</math></b>	<b>(SE)</b>	<b>F</b>	<b>P</b>	<b><math>\Delta</math>AIC</b>
DNA damage		-0.00803	(0.0458)	0.03	0.8609	
Exposure		0.0766	(0.0771)	0.75	0.3886	
Period	Spring 2014	0.0969	(0.2806)	<b>9.76</b>	<b>0.0004</b>	
	Fall 2014	<b>1.0561</b>	<b>(0.2836)</b>			
	Spring 2015	0				
Sex	Females ♀	0.0999	(0.0818)	1.49	0.2225	
	Males ♂	0				
Age	Adults	0.1116	(0.1453)	0.68	0.5076	
	Young adults	0.1018	(0.0933)			
	Juveniles	0				
Exposure*Sex	Females ♀	<b>-0.2210</b>	<b>(0.0933)</b>	<b>5.61</b>	<b>0.0185</b>	+2.7
	Males ♂	0				
Exposure*Age	Adults	0.1207	(0.1207)	1.42	0.2445	-4.8
	Young adults	0.1850	(0.1850)			
	Juveniles					

**Table 2.6.** Screenshots with the frequency plots depicting within-sample variation in a typical comet assay data of wild voles' WBC and a brief description of a typical profile.

Slide A	Slide B	Type of Comet Profile
		Type 0: Majority of nucleoids show comet tails that correspond to <5% of damage. Max damage is rarely >10%
		Type 1: a few nucleoids have comets showing >5% of tail DNA damage; 90 <sup>th</sup> percentile often falls between 35% and 55%
		Type 2: <50% of responded nucleoids with various sizes of comet tails
		Type 3: Mixture of intact and comet-resembling nucleoids
		Type 4: Majority of nucleoids with clear comets of various migration levels, however some rare intact nucleoids appear
		Type 5: All cells responded to comet assay moderate-to-high degree of damage



**Table 2.7:** Regression parameters modelling the log10-transformed estimates of the whole-body burden of cesium-137 as predicted variable and the environmental exposure rate as independent variable.

Sampling period	N	Intercept	$\beta$	$\beta$ error	R-squared
2014 Spring	98	<b>0.7591</b>	<b>0.8979</b>	0.1031	<b>0.4356</b>
2014 Fall	523	<b>2.52960</b>	<b>0.6848</b>	0.0472	<b>0.3181</b>
2015 Spring	275	<b>2.57045</b>	<b>0.88706</b>	0.0513	<b>0.5214</b>

**Table 2.8.** Difference of the least square means of the log10-transformed estimates of  $^{137}\text{Cs}$  body burden provided between sampling periods.

Comparison	Estimate	SE	<i>df</i>	<i>t</i> -stat	Pr>  <i>t</i>
2014 Spring – 2014 Fall	<b>-1.7688</b>	0.0696	855	<b>-25.42</b>	<b>&lt;0.0001</b>
2014 Fall – 2015 Spring	-0.0057	0.0743	855	-0.12	0.9921
2014 Spring – 2015 Spring	<b>-1.7745</b>	0.0475	855	<b>-23.89</b>	<b>&lt;0.0001</b>

## CHAPTER 3.

DNA DAMAGE, FLUCTUATING ASYMMETRY AND BODY BURDEN OF  
CESIUM-137 IN THE DOWNY EMERALDS (*CORDULIA AENEA*) OF CHERNOBYL<sup>13</sup>

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<sup>13</sup> Einor D., A. Golden, A. Bonisoli-Alquati & T.A. Mousseau, *to be submitted*

## ABSTRACT

Environmental effects of contamination by radionuclides are of great interest in ecotoxicology, however the risks of adverse physiological anomalies due to ionizing radiation remain under-explored. Here we report the preliminary results of a study between the markers of developmental instability and associated genetic damage in wild-caught species of dragonflies, *Cordulia aenea*, from the Chernobyl Exclusion Zone. Developmental instability was measured by looking at wing fluctuating asymmetry. Levels of genetic damage were approximated using the comet assay of the insects' eye tissue. We report no significant relationship between burden of radionuclides and DNA fragmentation in somatic cells. In a fully generalized mixed-effects model, elevated burden of radionuclides in individuals and high levels of DNA breakage were associated with low levels of developmental instability, which suggests a significant selection against phenodeviated individuals.

## INTRODUCTION

Developmental stability (DS) is described as even growth of identical cellular lineages in an organism under optimal circumstances (Graham *et al.*, 1993). Extensive evidence has shown that exposure to environmental stressors, if not buffered, may cause long-term consequences for individual development, and therefore influence DS (Markow, 1994; Møller & Swaddle, 1997). Despite extensive research, the mechanism of this is typically depicted as a black-box event. Random perturbations to development that occur during stress cause biochemical effects in many important genetic and physiological components of cells during their differentiation and thus cause changes of patterns of tissue growth (Polak, 2003; Klingenberg, 2003). A simple analysis of bilateral symmetry of the body parts, which in theory share an identical developmental algorithm, can reveal a broad spectrum of such patterns. In the biological literature, any unexpected random deviation from perfect symmetry is referred to as fluctuating asymmetry (FA, reviewed in Møller, 1997). FA, as a measure of developmental instability, has been proposed as a biomonitoring tool by Palmer and Strobeck (1986). Since then, the effects of various environmental stressors on the markers of individual development, including the FA, were extensively researched (Clarke, 1993; Palmer & Strobeck, 1997; De Coster, *et al.*, 2013). It has been demonstrated, that increased FA is associated with a decrease in individual fitness in many species (meta-analysis: Beasley, *et al.*, 2013) and, as such, a decrease in symmetry was found to be negatively correlated with mating success (Watson & Thornhill, 1994; Møller, 2002), fecundity (Hendrickx, *et al.*, 2003) and survival (e.g. flight performance: Hurtado, *et al.*, 1997; chance of predation: Galeotti, *et al.*, 2005). It was concluded that FA as a measure of

perturbation in DS provides an important proxy for fitness and life history of a study organism.

Ionizing radiation (IR) has been long recognized as a significant inducer of sporadic errors in the genome and a disruptor of physiological processes (Mettler Jr & Upton, 1995). “Natural experiments” in Chernobyl, Fukushima and other areas, accidentally contaminated with radioisotopes, provide opportunities to study the effects of a chronic mutagen on the development of organisms over many generations. Overall, high mortality rates, elevated genetic abnormalities and cancer incidences, indicators of a decreased fitness, have been attributed to the strong detrimental effects of IR (e.g. Kryshev & Sazykina, 1998; Wing, *et al.*, 1999; Yablokov, *et al.*, 2009; Møller & Mousseau, 2007, 2009, 2013). Compared to other biomarkers, the relationship between LDIR and the magnitude of FA in animals has not been well investigated. In fact, various invertebrates, such as insects and other arthropods, have become convenient study models of the IR effects due to their ease of sampling and symmetry analyses. For example, in leaf beetles, *Chrysomelidae*, (Medvedev, 1996), stag beetles, *Lucanus cervus* (Møller, 2002), and two species of burying beetles (Trofimov, 2014), a positive association between IR and FA was reported. At the Fukushima Exclusion Zone, an experimental trial found increased incidence of bilateral abnormalities (Hiyama, *et al.*, 2012, 2013). Contrary to the above findings, it was reported in a different study that parental irradiation by the contaminants from Chernobyl was not associated with FA in the offspring of grasshoppers, *Chorthippus albomarginatus*, reared in common garden conditions (Beasley, *et al.*, 2012). Additionally, water crustaceans, *Asellus aquaticus*, which were sampled from the radioactively contaminated pond near the failed

reactor of Chernobyl NPP, did not exhibit any noticeable effects of contamination on the levels of FA (Fuller, *et al.*, 2017).

At the same time, the relationship between FA and IR in the vertebrate species has been studied much less extensively. For example, barn swallows, *Hirundo rustica*, were reported to have increased tail asymmetry and more frequent incidences of partial albinism in the contaminated areas of the CEZ (Møller, 1993; Ellegren, *et al.* 1997; Møller & Mousseau, 2001; Møller, *et al.* 2007). As well, increased FA of the skull was reported in the yellow-necked mouse, *Apodemus flavicollis*, affected by Chernobyl (Oleksyuk, *et al.*, 2014). Similar patterns of increased asymmetry in response to LDIR were found in plant research as well (Zakharov & Krysanov, 1996, Ivanov, *et al.*, 2015; Boratyński *et al.*, 2016). Uncertainty remains, however, between the genetic and physiological components of the manifestation of radiation-related developmental abnormalities. In other words, it is still unknown to what extent the IR-induced instability in cellular functions contributes to variations in tissue growth (Møller & Swaddle, 1997; Beasley, *et al.* 2012).

In this study, we studied the wild downy emerald dragonflies (*Cordulia aenea*, *Corduliidae*, *Odonata*) that inhabit areas of the CEZ under varying levels of ambient radioactivity. We aimed to test whether the insects affected by radio-contamination exhibit genetic damage from LDIR, and whether that was related to markers of developmental instability. We tested preserved tissue samples of these insects for levels of chromatin fragmentation in the somatic nuclei using the comet assay. The comet assay allows the investigation of DNA breakage on the level of a single cell. Asymmetry levels were measured in the left and right hindwings of these insects. We hypothesized that IR would induce genetic damage that would be reflected in the violation of the DS. Consequently, our

expectation was that DNA fragmentation levels would positively relate to estimated burdens of radioactive isotopes as well as to markers of developmental instability, namely the FA.

## MATERIALS AND METHODS:

### *Animal collection and dosimetry*

An expedition to the Chernobyl Exclusion Zone (CEZ) was conducted in June 2013. Sampling sites are shown on Figure 3.1. We used hand-nets to capture dragonflies, 37 of which were identified as *Cordulia aenea*. We selected this species as it was likely to be captured with the same success rate across the various areas of the CEZ. Insects were terminally anesthetized and put into vials containing 300  $\mu$ l of tissue preservative (RNAprotect, QIAGEN). Wings were gently removed from the body prior to preservation. The tubes were then immediately cooled down to +4C and then frozen at -70C upon being transferred to the University of South Carolina, Columbia for further processing.

Rate of ambient environmental radioactivity was measured via pre-calibrated handheld dosimeters (Inspector, SE International, Inc. Sebastopol, CA, USA) at 1cm above the ground-level. Measurements were taken several times and then averaged to a point estimate in  $\mu$ Sv h<sup>-1</sup>. The Red Forest and the village of Vesnyane were selected as high radiation experimental sites, as the near-ground exposure rate ranged between 8.7 and 14.4  $\mu$ Sv/h with an average of 10.1  $\mu$ Sv h<sup>-1</sup>. Additionally, two insects were caught inside the evacuated city of Pripjat, where the average exposure rate was estimated at 2.3  $\mu$ Sv h<sup>-1</sup>. Control sites were chosen near the villages of Yampol and Roz'izdzhe, and at the southern border of the CEZ near the Dytyatki village. In all three areas, the exposure rate was found

to be near normal background radiation levels for this region of the world (0.1-0.15 uSv/h). Overall, this provided a 20- to 100-fold difference in radiation dose rates between control and experimental sites.

All body burden measurements were determined from the dry mass of each individual insect (without accounting for the tissue sampling for the comet assay). Internal activity of radioisotopes was determined using a pre-calibrated Berkeley Nucleonics SAM940 radionuclide identifier system, equipped with 10x10x10 cm NaI detector, which was housed in about 600kg lead shielding to minimize background radioactivity within the measurement chamber. Because of the very low dry mass (<0.15g), gamma spectra acquisition required 24h per individual insect. Obtained spectra were compared to background reference spectra taken repeatedly over the course of measurements. Cesium-137 activity was estimated by integrating the activity above the background threshold at a specific signal of 662 keV, which corresponds to the energy of the gamma photons of the cesium-137 decay products. The resulting data were then converted to estimates of the relative activity of <sup>137</sup>Cs, standardized across samples by individual mass. To avoid the use of zeros during log-transformation, 0.1 was added to all measurements of the cesium-137 body burden.

#### *Wing measurements*

Wings were flattened by placing them between two 0.8mm glass slides without fixation and scanned using an Epson V300 Photo Color tabletop scanner at a resolution of 2400 dpi. A total of 17 landmarks were located at vein intersections and termination points (Fig. 3.3). The landmarks were anatomically homologous among all individuals, fulfilling



the criterion of Type I landmarks (Bookstein, 1991). Wings were scanned only once, therefore no measurement error analysis could be provided. However, previous wing asymmetry analyses, which used the same scanning technique, showed that the variation in shapes between the left and right wings exceeded measurement error due to scanning or digitizing by a factor of 3. (Beasley, *et al.*, 2012). Landmarks were digitized using the software TpsDig2.16 (Rohlf 2005). Subsequently, Procrustes coordinates for each landmark were obtained with the software MorphoJ, v.1.06d (Klingenberg, 2001), which also allowed for extraction of shape and size information from the raw data. A full Procrustes fit was used to account for wing orientation and Procrustes ANOVA was used to calculate centroid size. The centroid size for the left and right hindwings was estimated as a square root of the sum of inter-landmark distances – which geometrically is a center of mass of the landmarks. (Klingenberg and McIntyre 1998). The difference between centroid sizes was used as a measure of the wing size FA. An unsigned measure of asymmetry was estimated as the ratio of an absolute difference between centroid sizes ( $|L-R|$ ) and their average  $((L+R)/2)$  (Palmer 1994).

#### *The Comet Assay:*

For the comet assay, eye tissue of dragonflies was chosen. Conveniently, it is one of the largest external organs in these insects. Also, albeit structurally different, the eyes of the vertebrate and invertebrate organisms both have poor regenerative abilities. In hemimetabolous insects, the functional parts of their compound eyes usually finish their development during the early nymph instars, and do not undergo any further changes (Friedrich, 2003). In mammals and birds, radiation-induced ocular injuries, which are

usually manifested as various forms of cataract due to coagulation of the intraocular proteins, are well-known effects of LDIR. (Jacob, *et al.*, 2012; Mousseau& Møller, 2013; Boratynski *et al.*, 2014; Lehmann *et al.*, 2016). Therefore, we expected similar radiosensitive properties for the insect eye, with a radiation effect being reflected in the DNA damage.

Tissue samples were taken from preserved insects. The tissue was gently homogenized in cold 1x PBS solution and processed in the neutral comet assay, per recommendations by Todoriki *et al* (2006) as follows. We used the “classic” method as proposed by Singh *et al.* (1988) with minor modifications. Slides for the comet assay were precoated with 1% normal-grade agarose one day before experiment. An aliquot of the eye tissue homogenate was resuspended in 1% low-melting point agarose at 38°C and then the mixture was pipetted onto the slides. For each sample suspension, two replicates were made with opposing positioning of the mixture droplet on each slide. Each droplet was immediately covered with a 22x22 mm coverslip. After coverslips were placed, the slide batch was moved to the dark incubator at +4°C and was briefly stored to allow the cell suspension in the agarose to solidify. After the gel solidified, the glass coverslips were carefully removed and the slides were placed into vertical glass staining jars containing lysis buffer (2.5M NaCl, 0.1M EDTA-Na<sub>2</sub>, 0.01M Tris, pH 10.01 with freshly added 1% Triton-X100) and left overnight in a temperature controlled incubator at +4°C. The staining jars were gently shaken over time to allow even diffusion of the lysis buffer. All subsequent steps were conducted under dim light conditions using ice-cold buffers to minimize experimental error.

After incubation overnight, slides were removed from the lysis buffer and gently washed with 1x TBE buffer (Tris-Cl pH 8.3, Boric acid, EDTA-Na<sub>2</sub>). They were then placed into an electrophoretic rig containing the same solution. Slides were haphazardly positioned in the electrophoresis rig, at least 1cm away from the sides, and aligned towards one of the electrodes. An electric current was then applied at 0.7V/cm for 20 minutes. Following this, the slides were quickly rinsed with a Tris buffer (0.3M Tris-Cl pH 7.49) and then fixed in 75% ethanol for 15 minutes.

The slides were stained with SYBR® Gold Nuclear Acid Gel Stain by applying the working solution (1:30,000) directly onto each of the dry agarose squares on the slides and spreading the droplet with a coverslip. Slides were left for 5 minutes and then washed with deionized water and left for dry. Images of the nucleoids were captured using a Zeiss Axioskop D40 microscope at 40x magnification equipped with an AxioCam HRM camera with the pre-installed AxioVision software package (Carl Zeiss, Inc., Thornwood, NY). Ratio of the fluorescence of migrated DNA over total DNA was used as an estimate of DNA breakage. For the best point estimate, 50 comet images from random areas were manually captured for each replicate slide (with total being 100 comet images) using CaspLab v1.2.2 software (Konza, 2001).

### *Statistical Analyses*

The comet assay data were summarized per each sample (Lovell, *et al.*, 1999) and log-transformed in accordance with recommendations by Bright, et al (2011). Median estimates were used as a central tendency measure of a comet distribution. The data were analyzed using simple linear regression and generalized linear mixed-effects models via

SAS statistical software ver. 9.3 applying PROC REG and PROC MIXED with sampling location in a RANDOM statement to account for possible effect of area of collection (SAS Institute Inc., Cary, NC, USA). Plots were performed using R package *ggplot2* (Wickham 2009). We tested the hypothesis that DNA damage would be associated with the burden of cesium-137 by fitting the model, shown in Equation 3.1.

$$\% \text{ DNA in comet tail} = \text{Burden} + \text{Sex} + \text{Sex} \times \text{Burden} \quad \text{Equation 3.1}$$

Only those individuals, for whom both wings could be measured, were used in the analysis. Seven dragonflies were found to have damaged wings, which made them unsuitable for landmark acquisition. Thus, these samples were excluded, reducing the total sample size to 30. We tested for directional asymmetry (specific developmental bias towards one of the sides) by performing a one-sample t-test for the left-to-right difference in centroid sizes, which was found to be non-significant ( $t_{29}=1.77$ ;  $P<0.087$ ). Normal distribution of the signed centroid size differences confirmed absence of antisymmetry (non-specific developmental bias towards either side) of the wings (Kolmogorov-Smirnov test:  $d=0.01$ ;  $P>0.200$ ; Shapiro-Wilk test:  $W= 0.97$ ;  $P<0.432$ ). We used the linear mixed-effects model (Equation 3.2) to predict the magnitude of FA (using the unsigned normalized centroid size difference) from the levels of DNA damage and the radiation dose approximated from the cesium burden.

$$\text{Fluctuating asymmetry} = \% \text{ DNA in comet tail} + \text{Burden} \quad \text{Equation 3.2}$$

## RESULTS

The sampled dragonflies, wild downy emeralds *Cordulia aenea*, used in the present report, included: (1) 16 insects (10 males; 6 females) that were captured in the three locations, characterized by high levels of contamination by cesium-137 – the city of Pripyat; the Red Forest and the village of Vesnyane (Fig. 3.1); and (2) 14 insects (8 males; 6 females) that were caught in areas previously recorded as not significantly contaminated by  $^{137}\text{Cs}$  – near the evacuated villages of Yampol and Roz'izdzhе, and near the Dytyatki CEZ control outpost (Fig. 3.1).

Our analysis of radiation body burden demonstrated that dragonflies from the areas, contaminated with radionuclides from the Chernobyl fallout, contained detectable levels of cesium-137 in the dry mass, whereas the spectra from insects collected from the control sites did not differ from the background readings (Fig 3.2a). Average relative activity of cesium-137 in dragonflies from the radioactive sites was estimated at  $8.39 \pm 2.42$  Bq per gram of dry mass (i.e.  $8390 \text{ Bq kg}^{-1}$ ), and ranged between  $1.36$  and  $30.32 \text{ Bq g}^{-1}$ . There was no difference in radioactive burden between the two sexes ( $F=0.47$ ;  $P<0.500$ )

The results of the linear mixed effect model (Equation 3.1) demonstrated that the radiation load, approximated from cesium burden, was not significantly associated with the levels of DNA breakage (Fig 3.2b, Table 3.1). Average comet DNA damage in the eye tissue samples from the insects was estimated at 25.77% and varied between 5.62% and 58.47%. We found no significant effect of sex on the levels of DNA breakage ( $F=0.14$ ,  $P<0.711$ ). However, significant interaction between sex and burden was detected ( $F=5.60$ ;  $P<0.028$ ), which was explained by opposing slopes of the prediction of DNA damage by each sex

(partial regression analyses revealed a non-significant negative slope for males,  $R^2=0.08$ ,  $F=1.24$ ,  $P<0.284$ , and a significant positive slope for females;  $R^2=0.32$ ;  $F=6.12$ ;  $P<0.033$ ).

Females had significantly larger wing size than males ( $F=9.96$ ,  $P<0.004$ ). The wing size was not associated with DNA damage ( $F=0.01$ ,  $P<0.932$ ), and it was not associated with the radiation burden ( $F=0.45$ ,  $P<0.509$ ). In simple linear regressions, the levels of the wing fluctuating asymmetry were not associated with the comet DNA damage ( $F=0.10$ ;  $P<0.750$ ; Fig 3.2c), or with the burden of radioactivity ( $F=0.15$ ;  $P<0.706$ ; Fig 3.2d). However, when included together in the full mixed effects model (Equation 3.2), the wing FA was negatively associated with both DNA damage and relative radioactivity levels (Table 3.2, DNA damage:  $F=5.01$ ,  $P<0.036$ ; Relative radioactivity:  $F=5.66$ ,  $P<0.027$ ).

## DISCUSSION

The aim of this study was to test for the genetic and developmental effects of the environmental stress by LDIR. We tested the relationship between the magnitude of the developmental instability, as estimated by wing size fluctuating asymmetry (FA), and genetic damage, estimated using the comet assay. We predicted that a higher radiation load would be associated with increased DNA breakage and higher levels of FA in wing sizes. However, our main finding was that the levels of DNA damage in the somatic tissue were not associated with the levels of accumulated cesium-137. This finding suggests that radionuclide body burdens in the insects in this region are not sufficient to result in DNA breakage, measurable by the comet assay. In previous research on adult insects, a significant increase in DNA damage was detected using the comet assay after the insect was

experimentally irradiated with at least 1 Sv of acute gamma radiation (e.g. Hasan *et al.*, 2006). In wild areas of the CEZ where the exposure rate is less than  $10\mu\text{Sv h}^{-1}$ , the cumulative dose of 1 Sv is unlikely to be absorbed by a flying insect, such as a dragonfly, throughout its life-span. However, the presence of the radioactive cesium in the insect body suggests that dragonflies might be affected by internal rather than environmental radiation. Future studies should aim to estimate the precise absorbed dose these insects can potentially encounter, by using the information on soil and water contamination by radionuclides, and by applying proper dose conversion coefficients (DCC; Cailes, *et al.* 2006; Garnier-Laplace, 2016).

Members of the *Corduliidae* family, like other dragonfly species, are obligate insectivores. For our study, this means that the intake of radioactive isotopes is significantly limited to the burden found in their prey. It is unclear, however, what part of the burden of radioisotopes might come from the contaminated lakes in which the dragonflies spend their early developmental stages (Murphy, *et al.*, 2011). Furthermore, a significant part of the life-time of these insects is spent mid-air or resting on top of a plant and thus at some distance from the most radioactive spots typically found in the soil. This perching behavior likely shields the dragonfly from surface radiation, thus decreasing the total absorbed dose. Nevertheless, the highest body burden was found in dragonflies caught in areas moderately and severely contaminated by radionuclides from the Chernobyl fallout. This finding clearly shows that radioactive isotopes such as cesium-137 are still circulating in the food chains of the ecosystems of CEZ. This is unsurprising, as only in June 2016 we passed the first half-life period mark for cesium-137, which is still the most abundant radioisotope in the CEZ. Absence of insects with a detectable burden of radioisotopes in the control areas

suggests low migration rate of the dragonflies away from their place of emergence, however more rigorous population surveys are required to test this hypothesis.

We found that the FA parameter was not predicted by DNA damage or cesium burden in simple linear regressions. However, when these variables were combined to predict the FA, we found that insects with increased DNA fragmentation levels and with high radioactive burden demonstrated lower magnitude of fluctuating asymmetry. Our finding supports our alternative hypothesis, which explains the result by the process of natural selection. Our data suggests that natural selection eliminates individuals with more asymmetric wings (allegedly due to increased DNA damage and physiological effects caused by radiation), perhaps due to a decrease in fitness. For wings this is especially crucial, since asymmetry affects the aerodynamic properties of the dragonfly, decreasing its abilities to efficiently maneuver, catch prey or defend its territory or mates. We did not establish the cause-effect relationship between radiation, DNA damage and FA. The fitness hypothesis in relation to the wing asymmetry and environmental contamination with radionuclides needs further testing. However, it was previously found that the populations of the red-eyed damselflies, *Erythromma najas* (*Odonata, Coenagrionidae*), from the contaminated lakes of the CEZ did not exhibit changes in wing FA due to radiation and did not show an increase in mutation rate (Cailes, 2006). The author concluded, that the LDIR did not have a significantly detrimental effect on the fitness of these insects. Follow-up studies should aim to collect larger sample sizes across as many sampling areas as possible, because rigorous and geographically diverse sampling are generally recommended in order to establish a reliable correlation between the FA and an environmental stressor (Palmer and Strobeck, 2003). Overall, broad spectrum of habitats, simplicity in wing landmark



acquisition for FA analyses and feasibility of genotoxicity tests in a single tissue provide strong foundation for further studies of the applicability of dragonflies in biomonitoring of the effects of environmental pollution, including contamination by radioisotopes.

In general, insects are known to be far less sensitive to low doses of IR than higher vertebrates, such as mammals and birds, which is explained by low cell turn-over rate in the late imago stages of insects (IAEA, 1992; O'Brien & Wolfe, 2015). Nevertheless, previous studies reported significantly decreased abundance of several orders of insects, including dragonflies, in the contaminated areas of the CEZ (Møller and Mousseau, 2009). Subsequently, variation in radiosensitivity between dragonfly species was found in the Fukushima exclusion zone (Møller, *et al.*, 2013) and it was suggested that the effects of radiation could be related to the ecological characteristics of this taxon. Decreased competition pressure from the more radio-sensitive dragonfly species could have promoted population growth of more radio-resistant ones. Therefore, because the dragonflies *C. aenea*, presented in this study, were found relatively abundant in the severely contaminated areas of the CEZ, it can be suggested that we have inadvertently chosen a species of dragonflies that might be highly resistant to IR. In other words, the choice of the study object may have biased the study in favor of seeing no effects.

The consequences of the nuclear accidents, like in Chernobyl, and more recently at Fukushima Dai-ichi, must receive stronger attention from the public and scientific community that will help understand and mitigate hazards that come from the inevitable radioactive pollution of our shared habitat.

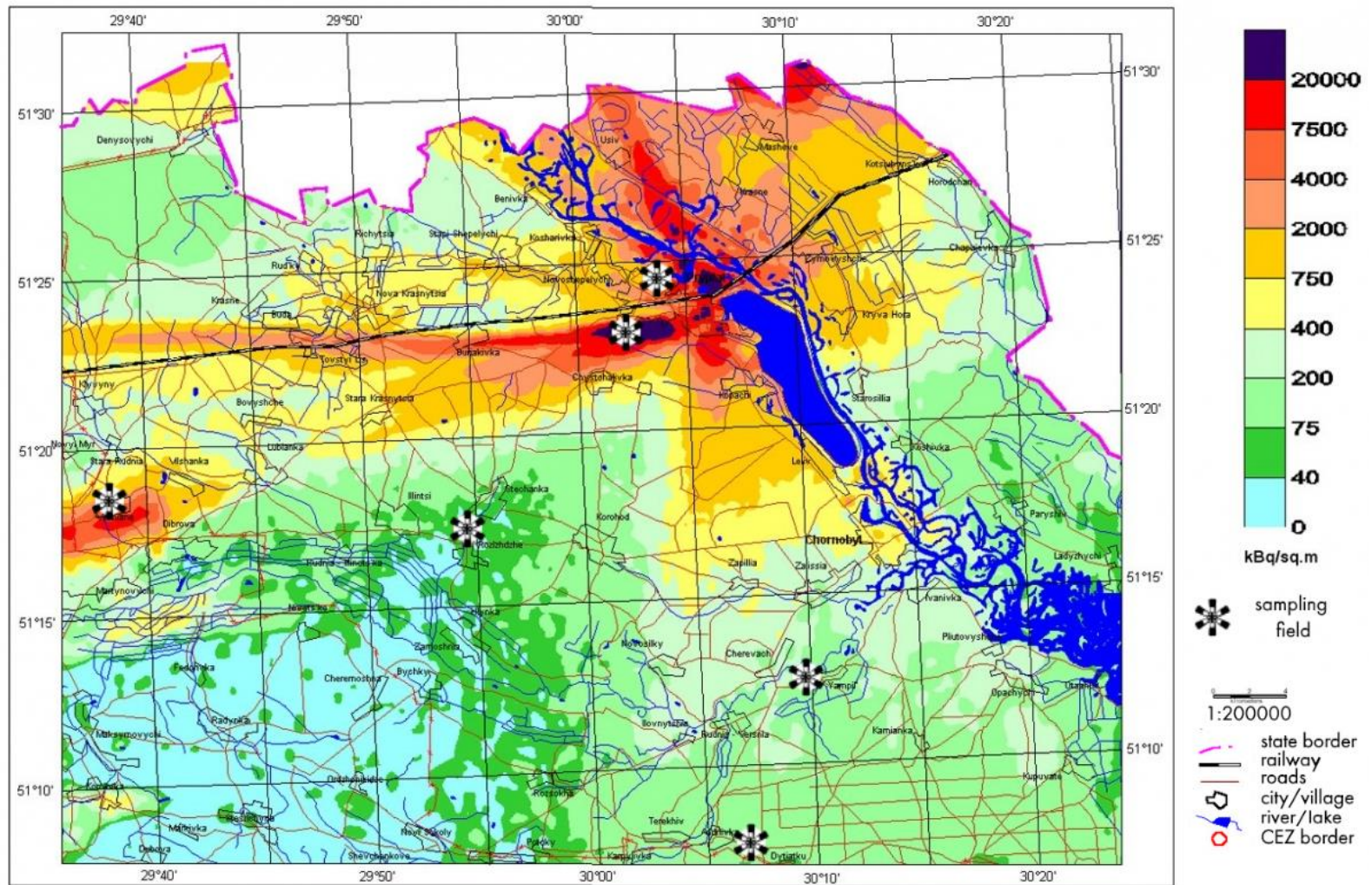


Figure 3.1. Map of the CEZ (showing levels of contamination by cesium-137 as of year 1997, source: radioecology-exchange.org) and sampling locations of the dragonflies.

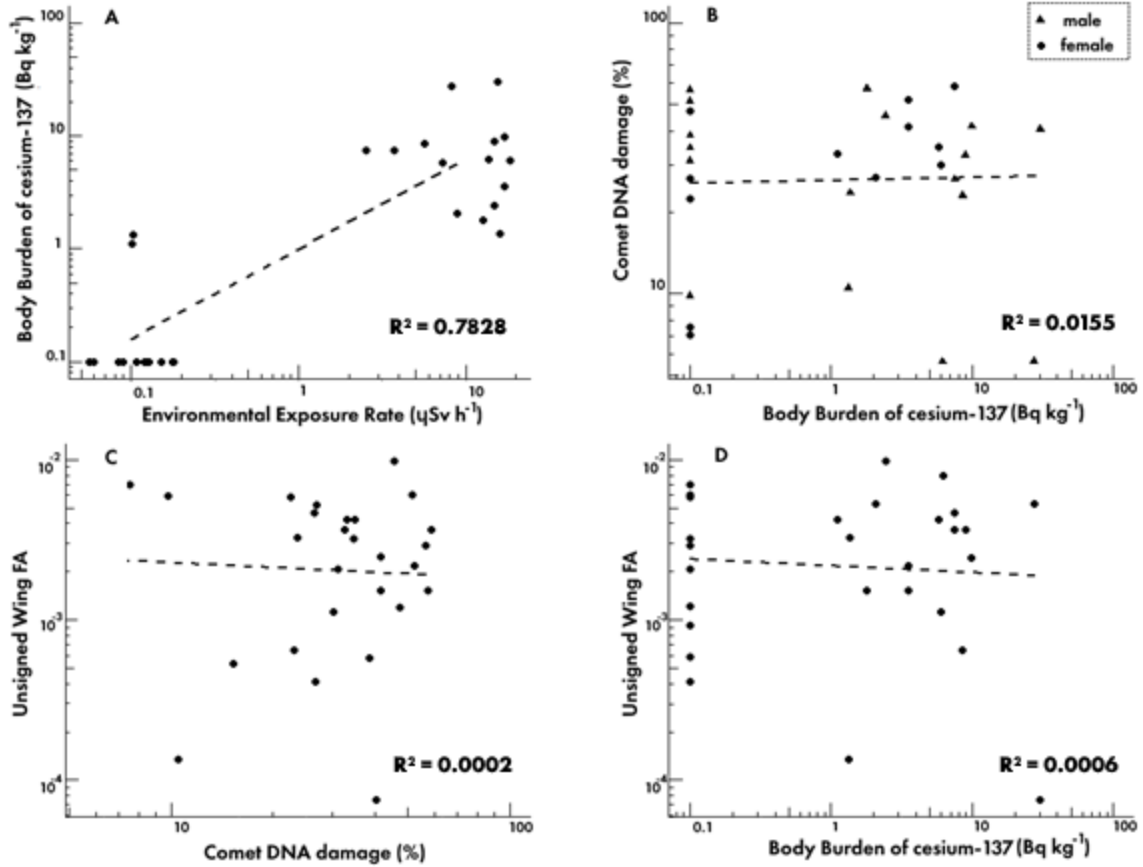


Figure 3.2 Scatterplots and linear associations that explore relationship between:

Figure 3.2a Rate of background radiation and burden of  $^{137}\text{Cs}$  in dry mass

Figure 3.2b Body burden of  $^{137}\text{Cs}$  in dry mass and levels of DNA damage measured in the somatic tissue of the dragonflies using the comet assay

Figure 3.2c Levels of DNA damage and unsigned normalized asymmetry of dragonflies' wings

Figure 3.2d Burden of  $^{137}\text{Cs}$  and unsigned normalized asymmetry of dragonflies' wings

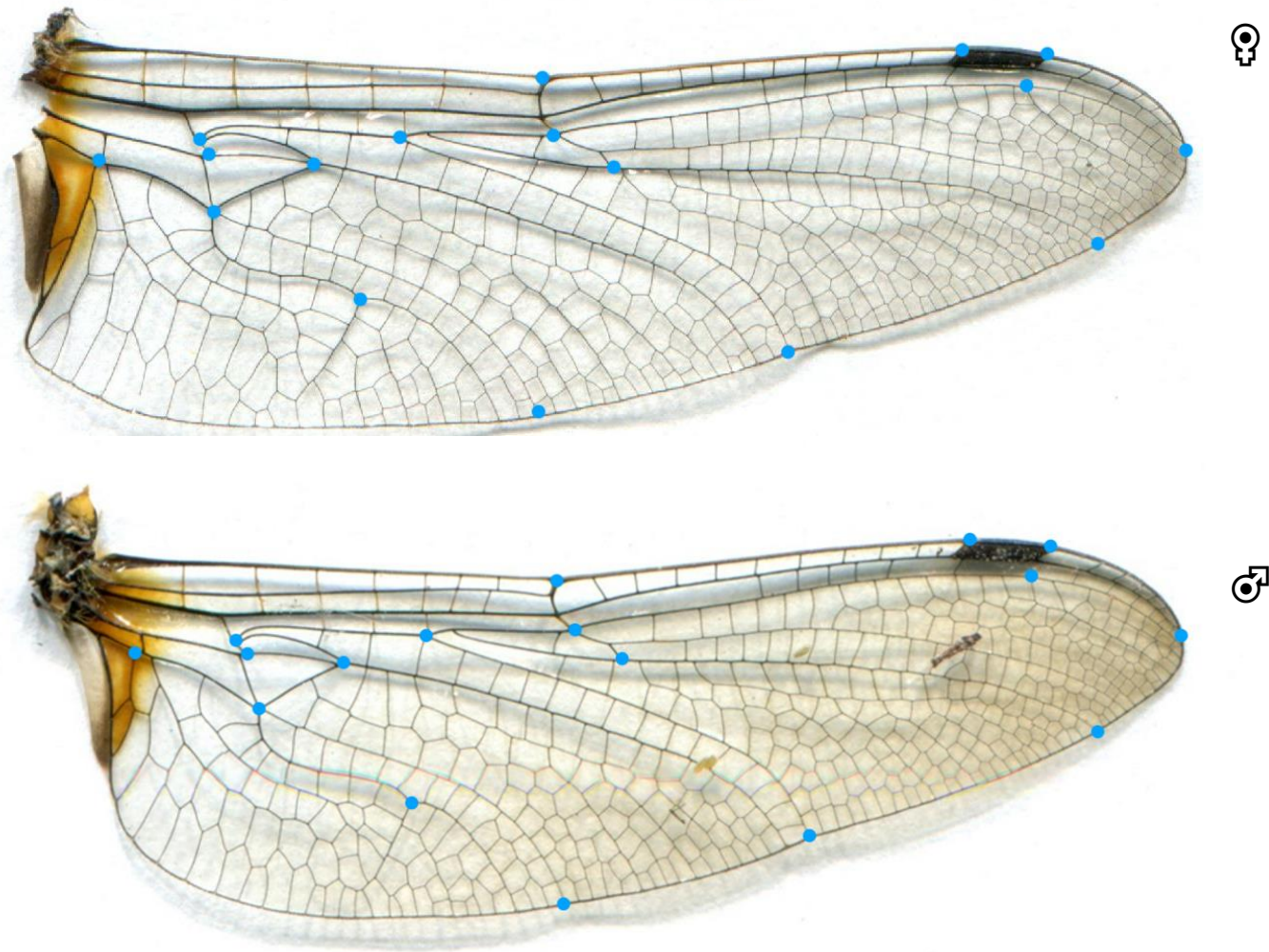


Figure 3.3 Placement of hind wing landmarks for female and male *Cordulia aenea*

**Table 3.1** Coefficients derived from the generalized linear mixed-effects model that predicts DNA damage from the burden of cesium-137 and sex of the dragonflies. The model had the following statistics: N = 30; *df* = 21; AIC = 67.4.

<b>Effect</b>	<b>Estimate</b>	<b>(SE)</b>	<b>df</b>	<b>F stat</b>	<b>P-value</b>
Cesium-137 burden	-0.096	(0.078)	21	0.86	0.2339
Sex	0.089	(0.260)	21	0.12	0.7344
Burden*Sex	0.316	(0.134)	21	5.60	<b>0.0276</b>

**Table 3.2** Coefficients derived from the generalized linear mixed-effects model that predicts magnitude of fluctuating asymmetry from the levels of DNA damage and radiation burden in the dragonflies. The model had the following statistics: N = 30; *df* = 22; AIC = 89.4.

<b>Effect</b>	<b>Estimate</b>	<b>(SE)</b>	<b>df</b>	<b>F stat</b>	<b>Pr &gt;  t </b>
DNA damage	-0.5327	(0.2380)	22	5.01	<b>0.0356</b>
Cesium-137 burden	-0.4024	(0.1692)	22	5.66	<b>0.0265</b>

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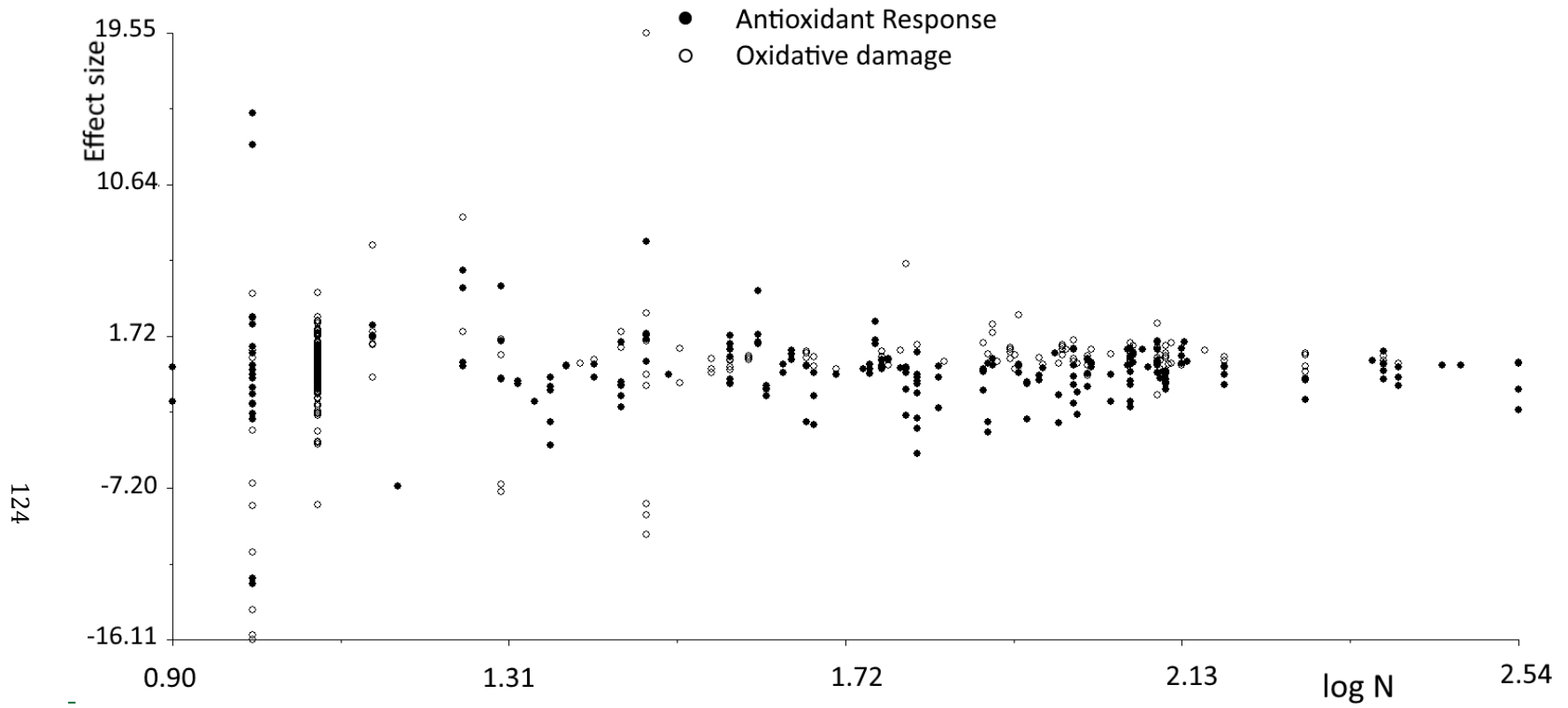
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## APPENDIX A

### SUPPLEMENTARY INFORMATION TO CHAPTER 1.



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Figure A.1. Funnel Plots for extracted Effect sizes versus total sample size.

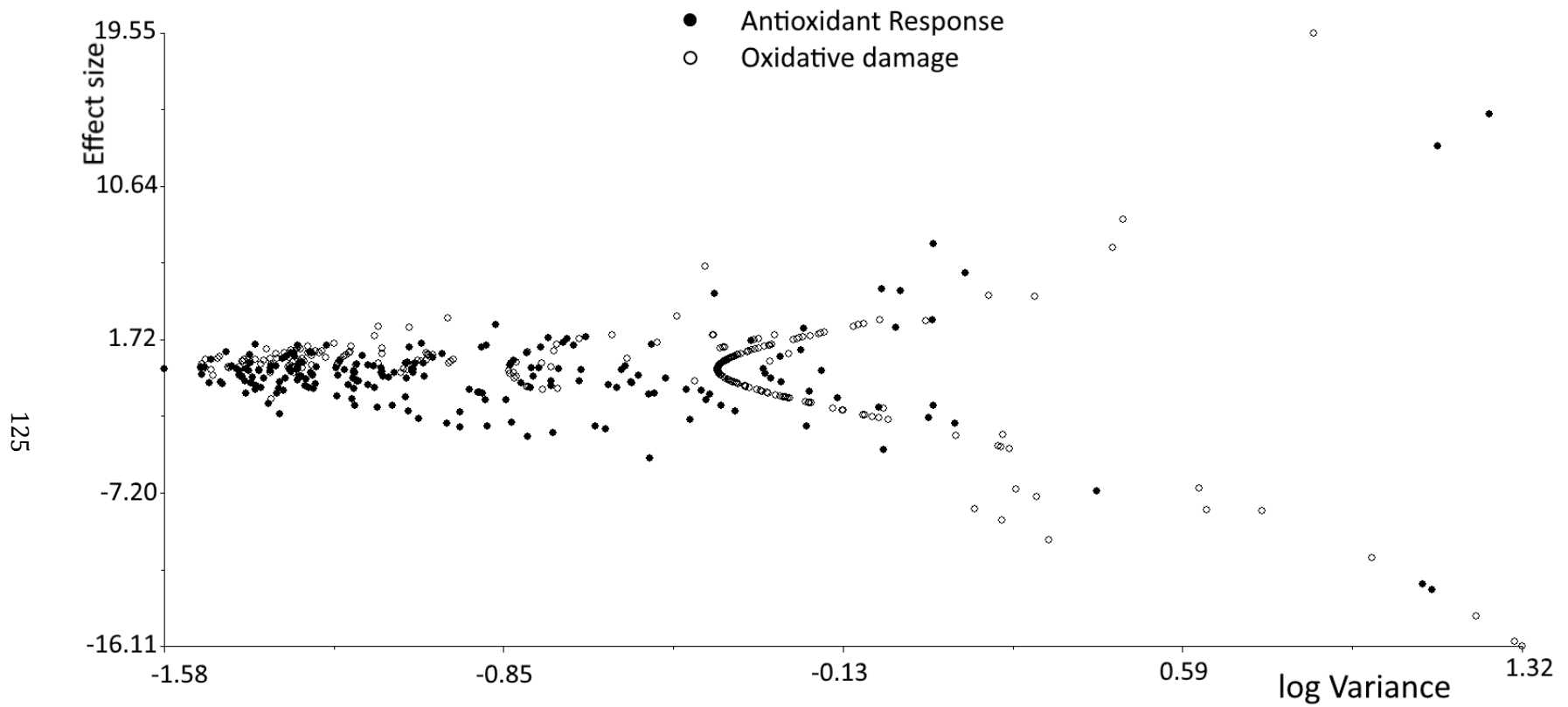


Figure A.2. Funnel Plots for extracted Effect sizes versus variance.

Table A.3. Description of the extracted papers.

Study#	Author	Year	Genus	species	Marker Type	Marker name
1	Ovsyannikova et al.	2001	Homo	sapiens	antioxidant	General AO activity β-galactosidase β-glucosidase CAT GPX Reduced Glutathione SOD
					oxidamage	MDA conjugates GSSG peroxide TBARS
2	Odinaev et al.	2001	Homo	sapiens	antioxidant	CAT
					oxidamage	MDA conjugates peroxide TBARS
3	Verhoglyad et al.	1991	Rattus	norvegicus	antioxidant	CAT
					oxidamage	MDA conjugates Free Fatty Acids
4	Tribrat et al.	1999	Homo	sapiens	antioxidant	CAT ceruloplasmin SOD
5	Bazhan et al.	1998	Homo	sapiens	antioxidant	CAT ceruloplasmin Erythrocyte Resistance

Study#	Author	Year	Genus	species	Marker Type	Marker name
					oxidamage	MDA conjugates TBARS
6	Drobinska et al.	1998	Homo	sapiens	antioxidant	SOD
					oxidamage	GSSG Peroxide Production TBARS
7	Voronkov et al.	1995	Homo	sapiens	antioxidant	CAT General AO activity
					oxidamage	Chemiluminescence MDA conjugates TBARS
8	Pasieshvili L.M.	1995	Homo	sapiens	oxidamage	MDA conjugates TBARS
9	Pasieshvili et al.	1994	Homo	sapiens	oxidamage	Chemiluminescence MDA conjugates TBARS
10	Pasieshvili et al.	1995	Homo	sapiens	oxidamage	Chemiluminescence MDA conjugates TBARS
11	Mirzoev et al.	1999	Bos	taurus	oxidamage	TBARS
12	Shishkina et al.	2005	Microtus	oeconomus	antioxidant	General AO activity
13	Ustinova et al.	2003	Apodemus	agrarius	antioxidant	CAT
					oxidamage	GSSG
			Clethrionomys	rutilus	antioxidant	CAT General AO activity
					oxidamage	MDA conjugates GSSG

Study#	Author	Year	Genus	species	Marker Type	Marker name
/continued/			Microtus	arvalis	antioxidant	CAT General AO activity
					oxidamage	MDA conjugates GSSG
14	Paranich et al.	1998	Rattus	norvegicus	oxidamage	MDA conjugates TBARS
15	Belov et al.	1997	Rattus	norvegicus	antioxidant	Erythrocyte resistance
					oxidamage	chemiluminescence TBARS
16	Moroz et al.	1997	Homo	sapiens	antioxidant	aosfactor CAT GPX GSH SOD
					oxidamage	MDA conjugates GSSG peroxide TBARS
17	Chayalo et al.	1997	Homo	sapiens	antioxidant	GPX GSH GT
					oxidamage	MDA conjugates GSSG TBARS
18	Antypkin et al.	1998	Homo	sapiens	antioxidant	CAT ceruloplasmin GPX

Study#	Author	Year	Genus	species	Marker Type	Marker name
/continued/						GSH
					oxidamage	peroxide TBARS
19	Abramova et al.	2005	Homo	sapiens	antioxidant	CAT GPX SOD
					oxidamage	MDA conjugates TBARS
20	Ovsyannikova et al.	2010	Homo	sapiens	antioxidant	AOS factor CAT SOD
					oxidamage	MDA conjugates
21	Champoux et al.	2006	Calonetrus	leucomelas	antioxidant	Vitamin A
22	Neronova et al.	2004	Homo	sapiens	antioxidant	CAR GSH SOD
					oxidamage	peroxide TBARS
23	Ivaniota et al.	1998	Homo	sapiens	antioxidant	CAT GPX GSH GST
					oxidamage	MDA conjugates GSSG Lipid Oxidation TBARS



Study#	Author	Year	Genus	species	Marker Type	Marker name
24 /continued/	Glushakova et al.	2000	<i>Mus</i>	<i>musculus</i>	antioxidant	Cytochrome b5 Cytochrome P450
25	Sidorik & Burlaka.	2000	<i>Rattus</i>	<i>norvegicus</i>	antioxidant	CAT SOD
26	Orehova &.	2011	<i>Apodemus</i>	<i>uralensis</i>	antioxidant	CAT
					oxidamage	TBARS
27	Lyashenko et al.	2000	<i>Homo</i>	<i>sapiens</i>	antioxidant	CAT SOD
					oxidamage	TBARS
28	Kashkalda et al.	2009	<i>Homo</i>	<i>sapiens</i>	antioxidant	GPX SOD
					oxidamage	Lipid Oxidation
29	Tereschenko & Kaskalda	2004	<i>Homo</i>	<i>sapiens</i>	antioxidant	GPX Vitamin A & Vitamin E
					oxidamage	TBARS
30	Yakovleva & Aleksievskaya	2010	<i>Homo</i>	<i>sapiens</i>	antioxidant	CAT SOD
					oxidamage	TBARS
31	Pelevina et al.	2011	<i>Homo</i>	<i>sapiens</i>	oxidamage	DNA damage ROS
32	Møller et al.	2005	<i>Hirundo</i>	<i>rustica</i>	antioxidant	Carotenoids Vitamin A Vitamin E
33	Møller et al.	2008	<i>Parus</i>	<i>major</i>	antioxidant	Carotenoids Vitamin A Vitamin E

Study#	Author	Year	Genus	species	Marker Type	Marker name
34 /continued/	Ben-Amotz et al.	1998	<i>Homo</i>	<i>sapiens</i>	antioxidant	Carotenoids Vitamin A Vitamin E
					oxidamage	MDA conjugates
35	Kumar et al.	2014	<i>Homo</i>	<i>sapiens</i>	antioxidant	General AO activity GSH SOD
					oxidamage	TBARS
36	Holloman et al.	2000	<i>Apodemus</i>	<i>agrarius</i>	antioxidant	CAT SOD
				<i>sylvaticus</i>	antioxidant	CAT SOD
			<i>Clethrionomys (Myodes)</i>	<i>glareolus</i>	antioxidant	CAT SOD
37	Ivanenko et al.	2005	<i>Homo</i>	<i>sapiens</i>	antioxidant	GSH
					oxidamage	GSSG
38	Souchkevitch & Lyasko	1997	<i>Homo</i>	<i>sapiens</i>	antioxidant	AOS factor CAT
					oxidamage	MDA conjugates TBARS
39	Jelka et al.	2005	<i>Bos</i>	<i>taurus</i>	antioxidant	SOD
					oxidamage	TBARS
40	Neyfakh et al.	1998	<i>Homo</i>	<i>sapiens</i>	antioxidant	Vitamin A

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**Author:** D. Einor, A. Bonisoli-Alquati, D. Costantini, T.A. Mousseau, A.P. Møller  
**Publication:** Science of The Total Environment  
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APPENDIX B. SUPPLEMENTARY INFORMATION TO CHAPTER 2.

SUCCESS OF VOLE TRAPPING IN FALL 2014 (N = 539) CORRELATED TO

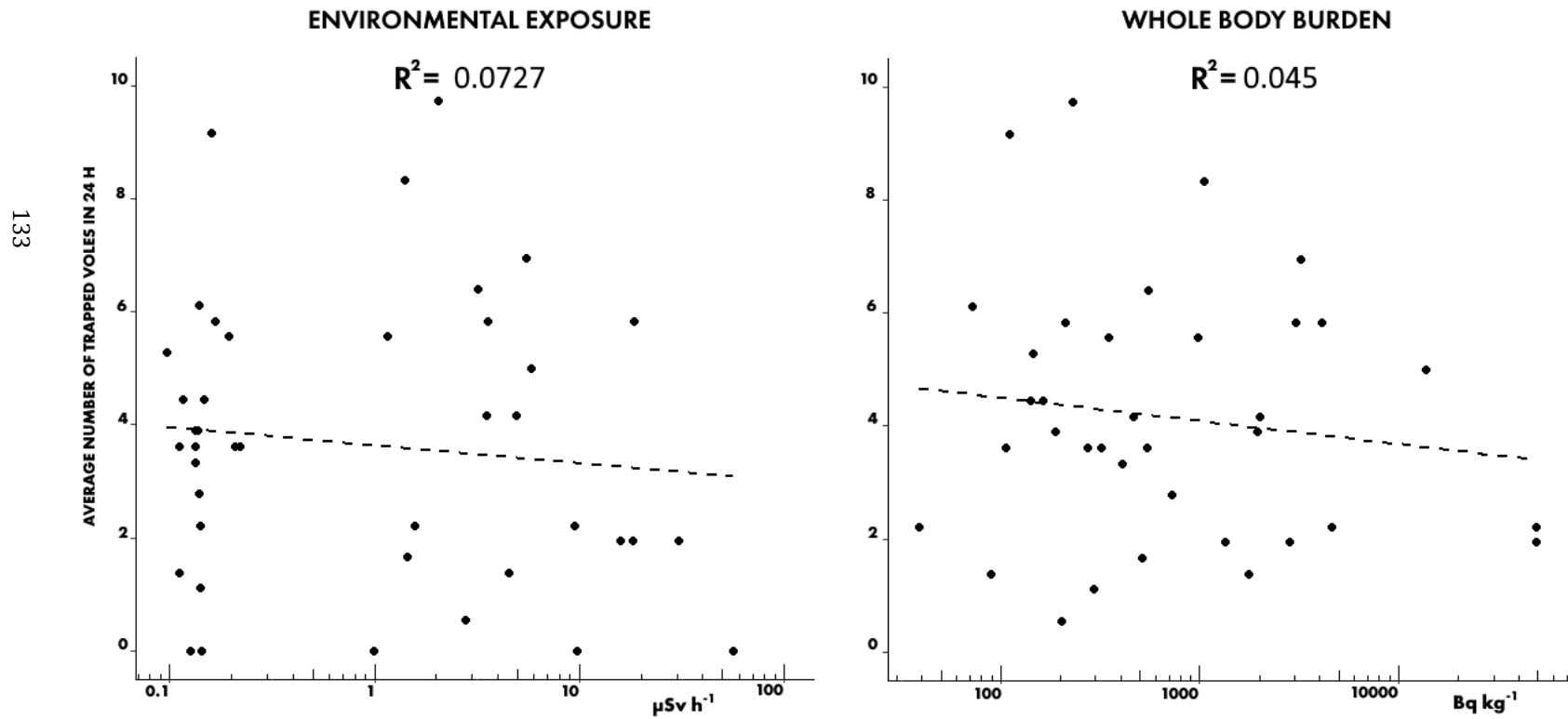


Figure B1 Rate of vole trapping in the CEZ, as a function of the radioactivity estimate