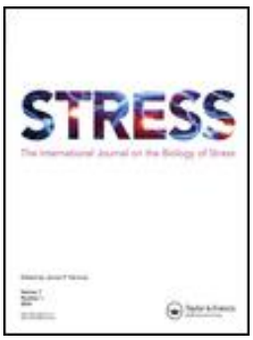

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ORIGINAL RESEARCH REPORT

Stress-associated radiation effects in pygmy wood mouse *Apodemus uralensis* (Muridae, Rodentia) populations from the East-Urals Radioactive Trace

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ABSTRACT

This work is based on the comparative analysis of data obtained in the course of monitoring pygmy wood mouse populations (*Apodemus uralensis* Pallas, 1811) in the East-Urals Radioactive Trace (EURT) area and background territories. The effect of population size and its interaction with the radioactivity on biochemical parameters in the spleen and adrenal glands was studied. The concentrations of total lipids, proteins, DNA and RNA, activity of glucose-6-phosphate isomerase and catalase as well as the level of lipid peroxidation (LPO) were evaluated. The functional-metabolic shifts seen with large population sizes were characterized by delipidisation of adrenocortical cells, increased LPO as the main mechanism for steroidogenesis, growth of the protein components of the adrenal glands to maintain their hyperfunction, as well as immunosuppression associated with the restriction of carbohydrates providing splenocytes, reduction of DNA synthesis, and the development of a pro-/antioxidant imbalance. Reactivity of the neuroendocrine and hematopoietic systems of animals experiencing a high population density was higher in the EURT zone compared with the reference group. This difference can be explained by the additional stress from the chronic radiation exposure. The level of LPO, catalase activity, and DNA/protein ratio in the spleen and the total protein content in the adrenal glands were the most sensitive to the interaction of population size and radiation exposure. The harmful effect (distress) of the interaction of non-radiation and radiation factors can manifest when there is a population abundance above 30 ind./100 trap–day and a radiation burden which exceeds the lower boundary of the Derived Consideration Reference Levels, which is above 0.1 mGy/day.

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Radioactive pollution; pygmy wood mouse; the EURT area; biochemical parameters; spleen; adrenal glands; stress in an overabundance population

Introduction

At the beginning of the mid-twentieth century, large areas of the northern hemisphere were contaminated by radionuclides as a result of nuclear weapons testing, normal operations, and accidents at nuclear-industrial complexes. Studies of the plant and animal populations living in areas of radioactive contamination are important for assessing the acceptable levels of chronic radiation exposure in populations of living organisms (Higley & Alexakhin, 2004; IAEA, 2002; ICRP, 2003).

On 29 September 1957, 74 PBq of radioactive waste was released into the environment as a result of an accident at the Mayak Plant (Southern Urals, Russia). As a result, a vast area (approx. 20,000 km^2) was contaminated. The radioactive contamination zone was called the East-Urals Radioactive Trace or EURT. Additional pollution of the plot in 1967 was due to separation of the radioactive sludge from the shores of Lake Krachai (UNSCEAR, 1993). Today, the main dose-forming radionuclides in the EURT zone are β-emitters ^{90}Sr and its daughter ^{90}Y, and β-γ-emitter ^{137}Cs, with the specific soil radioactivity of the latter being two orders of magnitude lower than that of $^{90}Sr + ^{90}Y$. Although many years have elapsed since the accident, the current radiation situation remains stressful in the most polluted parts of the EURT and is mostly determined by long-lived ^{90}Sr that is concentrated in the upper soil layers (Molchanova et al., 2014).

Currently, there is no doubt that ecological factors of non-radiation origin can modify radiation-induced biological effects (Mothersill & Seymour, 2009; Petin & Kim, 2014; Real et al., 2004). Such modifications with relatively small radiation doses, which occur at the boundaries of permissible measures and influence the population, are particularly noticeable. It is therefore important to study the mechanisms by which other factors either strengthen or weaken the effects of radiation. For a number of important physiological processes, such as the neuroendocrine and hemopoietic systems, the response to irradiation is not specific and is similar to effects caused by stress (Kondo, 1993). For mouse-like rodents, overpopulation represents a significant stressor on its own (Christian, 1963). Functional-metabolic characteristics of the spleen and adrenal glands are useful as the indices of nonspecific adaptive reactions to stress (Aguilera et al., 1996; Chen & Parker, 2004; Konduru, 2008; Satoh et al., 2006). We hypothesize that the factor of "relative abundance of mice" will modify the curves of the "dose-effect" produced for a number of functional-metabolic characteristics of the spleen and adrenal glands.

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Supplemental data for this article can be accessed here.

The aim of the study was to analyze the effect of population size and radiation dose on functional-metabolic changes in the spleen and adrenal glands of *Apodemus uralensis*, the pygmy wood mouse (Muridae, Rodentia), from the EURT zone.

For this purpose, the following tasks were set:

- comparative study of the effect of population size on biochemical parameters in mice from the reference and EURT areas;
- study of the effect of whole-body radiation dose rate on biochemical parameters in mice within the EURT area;
- assay of the interaction effect of "overpopulation" and "radiation dose rate": quantitative assessment of interactions of non-radiation and radiation factors;
- multivariate comparison of rodent samples: the role of environmental factors in the evaluation of radioactive effects on animal populations.

Materials and methods

Animal experiments were conducted at the Institute of Plant & Animal Ecology UB RAS (Russia) and approved by the local ethics committee.

Study locations and trapping methods

This work was based on the comparative analysis of data obtained in the course of monitoring the pygmy wood mouse populations in the Southern Urals from July to October 2010–2014. The impact study plots were located on the southwestern shore of Uruskul Lake (55°49′N, 60°53′E), 20 km from the epicenter of the accident at the Mayak Plant (Figure 1). The density of soil contamination with ^{90}Sr in that location is 3.3–22.3 MBq × m^{-2} (Atlas of the East Ural…, 2013). The reference (control) mice were caught in three areas located beyond the EURT zone, namely in the Chelyabinsk Oblast (55°47′N, 61°22′E) and Kurgan Oblast (54°22′N, 64°29′E; 54°47′N, 66°27′E). The level of soil contamination with ^{90}Sr in the reference regions is background for the Urals (0.3–3 kBq × m^{-2}) (Molchanova et al., 2014), and the ^{90}Sr-specific activity in bones of the reference mice is within a range of 0.2–0.6 Bq × g^{-1} (Starichenko et al., 2014).

Live-mouse traps with standard bait (bread and sunflower seed oil) for trapping rodents were used. The 10–25 traps were placed in a line at a distance of 3–5 meters from each other for a period of 2–10 days. Multiple lines of traps were placed on the study and reference plots.

The habitats characteristic for the trapping plots are shown in Table 1. It should be noted that, after the accident of 1957, the settlements on the territory of the central axis of the EURT were destroyed. The contaminated area was set aside as a protected area, and the areas used in our study were not engaged in any economic activity. There are no other similar areas close to the EURT. Therefore, as controls, we used animals (reference mice) caught in various habitats located in the areas with the background level of radiation contamination.

Object of study

The pygmy wood mouse (*Apodemus uralensis* Pallas, 1811) was used as the object of study. This rodent is one of the most numerous species of small mammals inhabiting the anthropogenically transformed areas of the Ural region (agrocenosis, forest plantations, fallows, gardens, areas transformed by chemical pollution, etc.) (Mukhacheva et al., 2010; Nurtdinova & Pyastolova, 2004). Similarly, on the ruderal meadow in the EURT area, this rodent is the most numerous of the small mammal fauna (Grigorkina et al., 2008). High numbers of pygmy mice within the EURT area can be located in birch groves if there are tier shrubs or dwarf shrubs (rose, cherry, raspberry) present.

One to four month immature mice (it was not possible to establish exact ages) with a weight of more than 10 g were used in this study. Their function is to preserve the population (a state of "preserved youth") until the next spring (Olenev, 2002). They were isolated by analysis of the teeth and reproductive systems (Klevezal, 2007). The study sample consisted of 113 individuals, and the reference population consisted of 70 individuals.

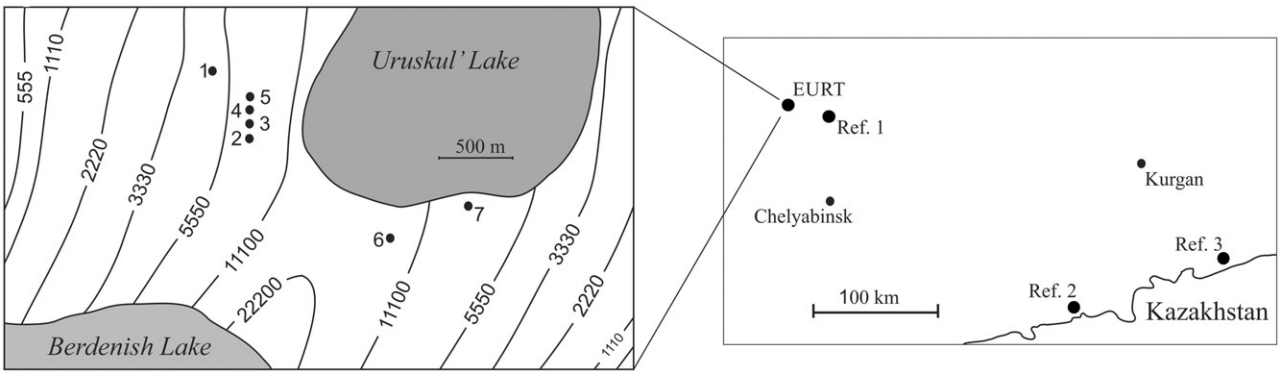

Figure 1. Trap placement. On the left – trapping sites on the EURT territory. Isolines show territory with contamination density of 90Sr (kBq × m^{-2}) according to (Atlas of the East Ural., 2013). On the right – layout of the study and reference plots, showing the boundaries of the Russian Federation (RF) and Kazakhstan, and the location of the regional centers of the RF.

Table 1. Characterization of trapping plots: periods of capture, relative abundance of mice, biotopic screening.

Area	Plots of trapping	Year of trapping	Month of trapping	Relative abundance of mice, ind./100 trap–day	Sample size for biochemical analysis, *n*	Characteristics of the biotope in which the animals were caught
EURT	1	2012	July	12	7	Thin birch park type with a significant amount of decomposed litter. Animal captures were confined to the wild rose bushes.
	2	2011		38	23	Located approximately 500 m west of the lake on the site of the village, after the formation of EURT resettlement. With the help of earthmoving machinery, all buildings constructed here were destroyed and buried in specially dug trenches. The vegetation cover is represented by ruderal areas of the community, with most of the vegetation comprising bromus inermis (*Cirsiumsetosum* (Willd.) Bess.), nettle (*Urticadioica* L.), wooly thistle (*Bromopsisinermis* (Leyss.) and bluegrass (*Poa*sp.). There were 2–5 plots removed from each other by a distance of 100–300 m.
		2012		48	7	
		2014		2	1	
	3	2011		32	21	
		2012		30	16	
	4	2011		12	7	
		2012		50	3	
	5	2011		4	4	
		2012		10	2	
	6	2012		8	2	The vegetation cover is represented by ruderal areas of the community, with most of the vegetation comprising bromus inermis (*Cirsiumsetosum* (Willd.) Bess.) and woolly thistle (*Bromopsisinermis* (Leyss.).
	7	2012		26	15	Birch copse with an admixture of aspen, located at 100–300 m from the lake. Uruskul. Animal captures are confined to the cherry bushes growing in the area.
		2014		12	5	
Ref. 1	8	2010	October	11	11	Birch-aspen groves with wild rose growing among the meadows and reservoirs. The distance between the plots was 100–500 m.
	9	2010		28	17	
	10	2010		6	1	
	11	2010		17	3	
	12	2010		6	1	
Ref. 2	13	2011	August	2	1	Abuttal between an agrocenosises covered with weeds.
	14	2012		24	4	Border of a meadow steppe and willow shrubs.
	15	2012		7	4	Border of a wheatfield and meadow steppe.
	16	2012		27	2	The ravine near a wheatfield.
	17	2012		65	12	Border of a mown hayfield and willow shrubs.
Ref. 3	18	2011		21	12	Waterlogged birch-aspen-willow grove in the field.
	19	2012		20	1	Steppe.
	20	2012		4	1	Wheatfield.

The method of calculating the relative abundance of the pygmy wood mouse

To calculate the relative abundance, the following formula was used:

$$N = (I/2 \times T) \times 100 \quad (1)$$

where N = the relative abundance of animals, I = number of pygmy wood mice collected in the first 2 days after setting traps, and T = the number of traps in the line. Thus, the relative abundance for each line of traps was calculated and represented as ind./100 trap–day (i.e. individuals per 100 trapped per day).

Based on our own observations and the long-term monitoring of mouse-like rodent populations in the EURT zone (Grigorkina et al., 2008; Grigorkina & Olenev, 2013), an abundance of animals from 1 to 15 ind./100 trap–day was considered a small population size, from 15 to 30 ind./100 trap–day was a medium population size, and above 30 ind./100 trap–day was a large population size.

Methods of biochemical studies

Within 15 min after death, the spleen and adrenal glands were weighed and frozen in liquid nitrogen; the organs were stored in a freezer at a temperature of −80 °C until further biochemical study in the laboratory.

During our biochemical studies, we measured mass rations: total lipids/protein, DNA/total protein, total RNA/DNA. The activity of enzymes glucose-6-phosphate isomerase (GPI) (EC 5.3.1.9) and catalase (EC 1.11.1.6) were analyzed. The level of lipid peroxidation (LPO) was estimated on the basis of the concentration of secondary products of LPO reacting with thiobarbituric acid reactive substances (TBARS).

The organs were homogenized in a Tris-HCl buffer solution (0.025 mol/L, pH 7.4) containing 0.175 mol/L KCl. The total lipids were extracted from the tissue homogenate with an ethanol:petroleum ether mixture (2:1). Nucleic acids (DNA, RNA) were extracted from the tissue homogenate by alkaline (0.3 mol/L KOH) and acid (0.5 mol/L $HClO_4$) hydrolysis (Dell'Anno et al., 1998).

Spectrometric methods were used to estimate the analyte quantities and the enzyme activities. The lipid test was performed with a vanillin solution (Fletcher, 1968), the protein test with Coomassie Brilliant Blue G250 (Kruger, 2002) and the TBARS test with thiobarbituric acid (Buege & Aust, 1978). The enzyme activity was determined through standard procedures using glucose-6-phosphate and resorcinol for GPI (Roe & Papadopoulos, 1954) and H_2O_2 and ammonium molybdate solution for catalase (Goth, 1991). Optical density measurements were performed using the microplate reader SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA).

The radiometric measurements

The ^{90}Sr-specific activity in the jaw was obtained via nondestructive β-radiometry, as previously developed. When switching from the β-particle count rate in the jaw to the ^{90}Sr + ^{90}Y-specific activity we used the following formula:

$$y = 16 \times x^{0.78} + 3 \quad (2)$$

Table 2. Radiation burden caused by ^{90}Sr and ^{137}Cs in animals trapped within the EURT zone.

Plots		Specific activity of a radionuclide in the soil layer 0–10 cm, Bq kg^{-1}[a]	External dose rate, mGy/day
1–5	^{90}Sr	35,537	1.06×10^{-7}
	^{137}Cs	958	0.006
6,7	^{90}Sr	98,210	2.90×10^{-7}
	^{137}Cs	5571	0.035

[a]Data are from Molchanova et al. (2009).

where y = specific activity of $^{90}Sr + {}^{90}Y$ in the jaw (Bq × g^{-1}) and x = β-particle count rate, normalized to the wet weight of the jaw (imp × s^{-1} × g^{-1}) (Malinovsky et al., 2012).

While the conversion coefficients from the β-particle counting rate to the $^{90}Sr + {}^{90}Y$-specific activity were obtained using the wet weight of bones, only the dry weight was known for the jaws from the depository. To account for the jaw drying, we applied a dry weight to a fresh weight conversion factor of 1.48, which was experimentally observed. On the basis of the work of Starichenko and Modorov (2013), it was decided that the $^{90}Sr + {}^{90}Y$-specific activity in the skeleton of a pygmy wood mouse from the EURT area was 78% of the specific activity in the jaw, and the ^{90}Sr-specific activity was half of the value obtained. For the calculation of the whole-body dose rate on the day of capture of the animals we used the conversion coefficient 1.5×10^{-6} (mGy/day) (Bq/kg of skeleton weight) (Malinovsky et al., 2014).

In order to estimate the external exposure of animals to ^{137}Cs and ^{90}Sr, the ERICA tool v. 1.2.1, Tier 2 was used (Brown et al., 2008, 2016). We used the following model parameters: the pygmy wood mouse, a terrestrial mammal which lives 20% of the time on the ground, and 80% of that time in the upper layer of the soil to a depth of 10 cm. The size of the animal was set at 2 × 2 × 8 cm.

The specific activity of ^{90}Sr and ^{137}Cs in the soil from the southern and western shores of Lake Uruskul was taken from the work of Molchanova et al. (2009). The average value of the index in the soil depths of 0–5 cm and 5–10 cm was used. It was thought that plots 6 and 7 (Figure 1) were located on the southern shore of Lake Uruskul, and plots 1–5 were located on the west coast. The analysis of the results is shown in Table 2.

When calculating the internal doses of ^{137}Cs, it was accepted that the specific activity of ^{137}Cs in the trapped rodents was 500 Bq × kg^{-1} (Tarasov, 2000). The animal radiation dose for a given specific activity was equal to 0.002 mGy/day. Note that the ERICA tool v. 1.2.1., Tier 2, and the approach we used to determine the internal dose of ^{90}Sr, gave similar results.

Statistical analysis

The calculations were performed using the STATISTICA vers. 8.0, STATGRAPHICS vers. 8.0 software packages and Past vers. 1.81. Mathematical processing of the data was performed by the analysis of covariance (ANCOVA), the regression analysis (simple and multiple linear regression), and the Mann–Whitney *U* test (StatSoft, 2012).

The dependence of the analyzed biochemical parameters (y) on the relative abundance of animals (x) was described by a linear regression equation as $y = b_0 \pm b_1 \times x$. The radiation dose-biochemical effect in the pygmy wood mice from the EURT zone, taking into account the population size, was described by multiple linear regression as $y = b_0 \pm b_1 \times x_1 \pm b_2 \times x_2$. For the pairwise comparison of the average values (M_1 and M_2) of the regression coefficients we used the Student's *t*-test. The calculated *t*-value was compared to the critical value, given a number of df ($n_1 + n_2 - 2$) and $p = 0.05$. The differences between M_1 and M_2 were determined to be statistically significant when the *t*-value ≥ critical *t*-value.

To take into account the interaction of categorical (reference/impact) and continuous (abundance of mice, ind./100 trap–day) predictors, the ANCOVA on the basis of the separate-slopes model was used. It was therefore possible to investigate the functional-metabolic effects of radiation in the small, medium, and large population sizes.

Multivariate analysis of the biochemical similarity of the rodent samples was performed by cluster analysis using the UPGMA algorithm and also the statistical test of bootstrapping (Efron, 1987) to assess confidence in particular internal branches of the tree.

The effect of the gender factor in the variability of biochemical indicators was assessed through the Mann–Whitney *U* test by comparing parameters in males and females trapped on one particular plot. The six plots of trapping (No. 9,7,3,2,17,18) used in comparison were balanced with respect to sexual composition. A significant difference between the males and females was not found (Supplement 1). Therefore, it was decided to unite males and females into a single group.

The abundance of *A. uralensis* in the Ref. 1 area varied from 6 to 28 ind./100 trap–day, from 2 to 65 ind./100 trap–day in the Ref. 2 area, and from 4 to 21 ind./100 trap–day in the Ref. 3 area. To justify the unification of reference mice from geographically remote plots of trapping, a comparison of Ref. 1, Ref. 2 and Ref. 3 was carried out by the ANCOVA (Supplement 2). We used the homogeneity-of-slopes model to test whether continuous predictor (abundance of mice, ind./100 trap–day) has different effects at different levels of categorical factor (Ref. 1/Ref. 2/Ref. 3). No interaction effect of categorical and continuous predictors was statistically significant at $p \leq 0.05$. No biochemical differences were evident between Ref. 1, Ref. 2, and Ref. 3. Therefore, it was decided to unite the reference mice into a single group.

Results

The effect of population size on the biochemical parameters is shown in Figure 2. The abundance of mice varied from 2 to 50 ind./100 trap–day in the EURT zone, and from 2 to 65 ind./100 trap–day in the reference areas. With the increase in the mouse population, the adrenal lipid/protein ratio was reduced, and the TBARS concentration and the total protein content were increased. In the spleen, the increase in the mouse population was correlated with a decrease in the total protein

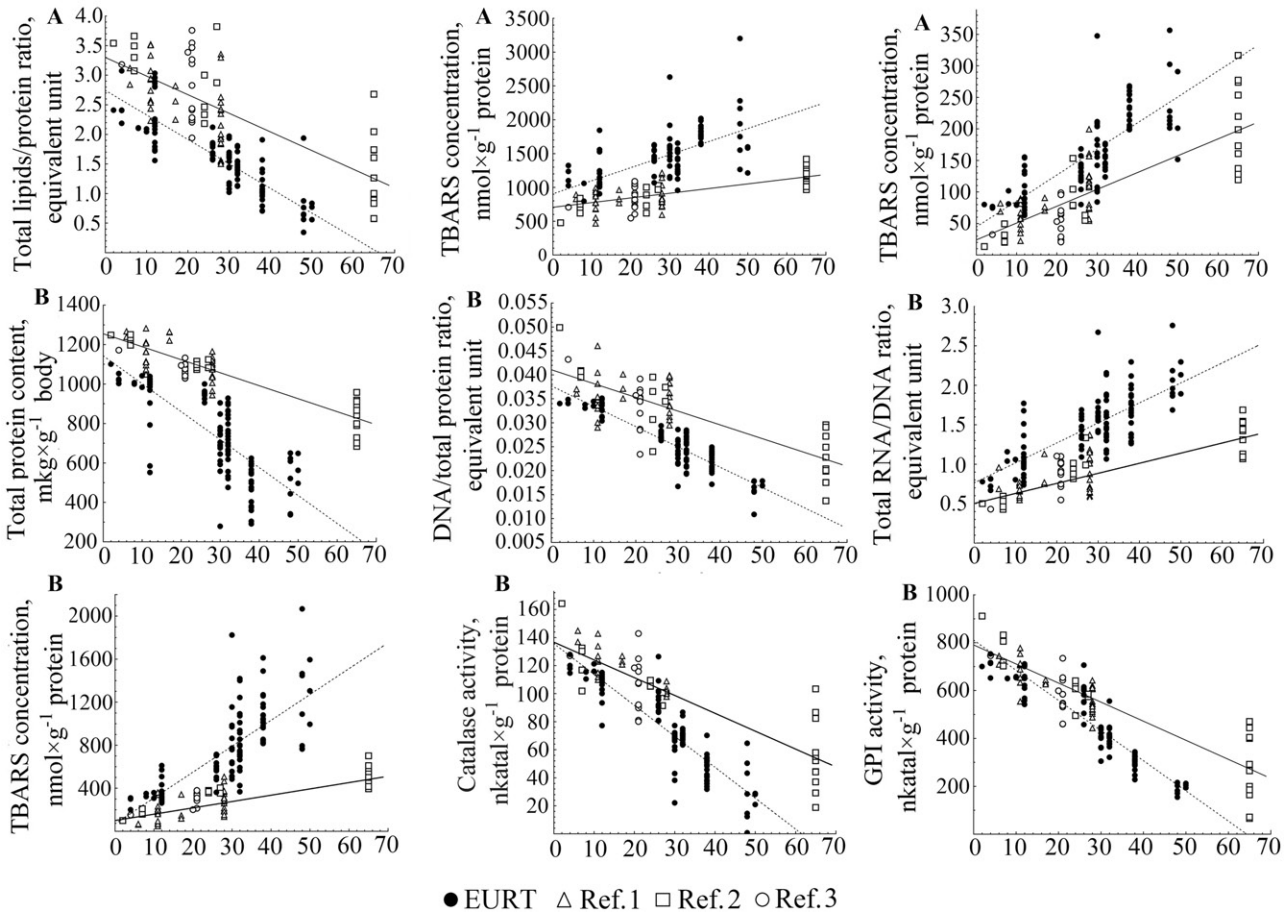

Figure 2. The effect of population abundance on biochemical parameters in *A. uralensis* from the reference and EURT areas. X-axis: relative abundance of mice (ind./100 trap–day). A – adrenal glands, B – spleen.

content, DNA/protein ratio, GPI and catalase activity, as well as with an increase in the RNA/DNA ratio and the TBARS concentration. These patterns were observed in the sample of animals from both the reference plots and the radioactively polluted plots.

The dependence of the analyzed biochemical parameters on the relative abundance of animals was described by a linear regression equation: $y = b_0 \pm b_1 \times x$. The results are shown in Table 3. For all equations, the 95% CI for b_1 coefficient values, obtained for the study and reference samples, did not overlap, and the absolute values of the b_1 coefficient in the samples from the contaminated area were always higher. In addition, the b_0 coefficient values for a number of parameters (TBARS concentration in the adrenal glands and the total protein content and the DNA/protein and RNA/DNA ratios in the spleen) in the study and reference samples had statistically significant differences.

The results of ANCOVA on the basis of the separate-slopes model are presented in Table 4. The interaction effect of categorical (reference/impact) and continuous (abundance of mice) predictors was statistically significant at $p \leq 0.05$. Because of this, with a small population size (10 ind./100 trap–day) the level of biochemical shifts in the study sample ranged from 1% to 65% relative to the reference values, whereas those from the medium population size (20 ind./100 trap–day) ranged from 9% to 111%. For large population sizes, the changes with respect to the reference group of animals were characterized as: 30 ind./100 trap–day by 20–143%, 40 ind./100 trap–day by 33–163%, 50 ind./100 trap–day by 53–178%.

The effect of the whole-body radiation dose rate on the biochemical parameters of the study animals is shown in Figure 3. The figure shows that the description of dependence by a linear equation is only possible if one ranks the study sample into two groups according to the population size: from 2 to 26 ind./100 trap–day (*a*) and from 30 to 50 ind./100 trap–day (*b*). Group *a* included mostly individuals trapped on plots 2 and 3, whereas in Group *a*, individuals prevailed from plots 1, 4, 5, 6, and 7. For both groups, the increase in radiation dose rate and the increase in population abundance had a similar effect on the biochemical parameters.

According to the regression analysis, obtained for Group *a* and Group *b*, the biochemical parameters can be described by multiple linear regression as $y = b_0 \pm b_1 \times x_1 \pm b_2 \times x_2$, where x_1 = whole-body radiation dose rate and x_2 = abundance of mice (Table 5). The biochemical shifts are more pronounced in the plots with a large population size: absolute values of b_1 and b_2 regression coefficients were higher in Group *b*. In Group *a*, the abundance values of mice (from 2 to 26 ind./100 trap–day) did not render a significant influence on the biochemical shifts: *t*-values for the b_2 coefficient were less than the critical $t_{0.05}$ value for five of the nine indicators.

Table 3. Effect of population abundance (x) on the biochemical parameters (y) in *A. uralensis* from the reference and radioactively polluted (EURT) areas: the results of simple linear regression $y = b_0 \pm b_1 \times x$.

		Reference area					EURT				
		df(n − 2) = 68					df(n − 2) = 111				
Parameter	Organ	β	$b_0 \pm$ SE	p	$b_1 \pm$ SE*	p	β	$b_0 \pm$ SE	p	$b_1 \pm$ SE*	p
Total lipids/protein ratio, equivalent unit	Adrenals	−0.73	$(33.2 \pm 1.2) \times 10^{-1}$	$<10^{-6}$	$(31.5 \pm 3.5) \times 10^{-3}$	$<10^{-6}$	−0.84	$(27.4 \pm 7.7) \times 10^{-1}$	$<10^{-6}$	$(41.5 \pm 2.5) \times 10^{-3}$	$<10^{-6}$
TBARS concentration, nmol × g^{-1} protein		0.68	**687.7 ± 32.3**	$<10^{-6}$	**7.6 ± 1.0**	$<10^{-6}$	0.61	**943.0 ± 75.4**	$<10^{-6}$	**19.1 ± 2.4**	$<10^{-6}$
Total protein content, mkg × g^{-1} body		0.78	25.0 ± 8.3	0.004	**2.7 ± 0.2**	$<10^{-6}$	0.78	44.5 ± 9.8	$<10^{-4}$	**4.1 ± 0.3**	$<10^{-6}$
Total protein content, mkg × g^{-1} body	Spleen	−0.87	**1238.3 ± 13.9**	$<10^{-6}$	**6.2 ± 0.4**	$<10^{-6}$	−0.76	**1143.4 ± 34.9**	$<10^{-6}$	**14.2 ± 1.1**	$<10^{-6}$
DNA/total protein ratio, equivalent unit		−0.76	$(41.0 \pm 0.9) \times 10^{-3}$	$<10^{-6}$	$(28.5 \pm 3.0) \times 10^{-5}$	$<10^{-6}$	−0.91	$(37.7 \pm 0.5) \times 10^{-3}$	$<10^{-6}$	$(42.4 \pm 1.8) \times 10^{-5}$	$<10^{-6}$
Total RNA/DNA ratio, equivalent unit		0.76	$(58.5 \pm 4.1) \times 10^{-2}$	$<10^{-6}$	$(12.4 \pm 1.2) \times 10^{-3}$	$<10^{-6}$	0.74	$(76.3 \pm 6.6) \times 10^{-2}$	$<10^{-6}$	$(25.3 \pm 2.1) \times 10^{-3}$	$<10^{-6}$
TBARS concentration, nmol × g^{-1} protein		0.82	129.7 ± 18.5	$<10^{-6}$	**6.6 ± 0.6**	$<10^{-6}$	0.76	69.7 ± 61.5	0.259	**24.0 ± 2.0**	$<10^{-6}$
Catalase activity, nkatal × g^{-1} protein		−0.86	138.2 ± 3.1	$<10^{-6}$	$(12.9 \pm 0.9) \times 10^{-1}$	$<10^{-6}$	−0.88	135.7 ± 3.4	$<10^{-6}$	$(22.0 \pm 1.1) \times 10^{-1}$	$<10^{-6}$
GPI activity, nkatal × g^{-1} protein		−0.87	771.7 ± 17.4	$<10^{-6}$	**7.7 ± 0.5**	$<10^{-6}$	−0.93	810.7 ± 14.5	$<10^{-6}$	**12.6 ± 0.5**	$<10^{-6}$

β (beta): standardized regression coefficient; df: degrees of freedom. *The values of the regression coefficients are given by modulus.
Bold font: differences between values (b_0 Reference – b_0 EURT; b_1 Reference – b_1 EURT) are significant for the use of $p = 0.05$.

Discussion

The changes in biochemical parameters of A. uralensis *depend on the level of population abundance: a description of the functional-metabolic effects of the neuroendocrine and hematopoietic systems*

Uniformity of the shift in biochemical parameters in study and reference mice (Figure 2) based on population abundance suggests the implementation of adaptive functional-metabolic reactions to stress factors independent of radiation exposure. The homeostasis of the population is based on the physiological mechanisms of the adaptive syndrome (Christian, 1963). During a population peak, when animals begin to compete more fiercely over environmental resources, a state of stress develops. This not only intensifies basic physiological systems through adrenocortical activity but also modifies the lymphatic and immune systems. The sequence of events that, in our opinion, leads to the development of the adaptation syndrome is given below.

Among the stereotypical morpho-functional changes, there is a reduction in lipid saturation of the adrenocortical cells as a result of internal steroidogenesis induction (Koldysheva et al., 2005; Molodykh et al., 1999). In our study, delipidisation of the adrenocortical cells occurred with increasing population size, along with a decline in the lipid/protein ratio in the adrenal gland, whereas the increase in the TBARS concentration indicates an intensification of LPO as the main mechanism for the mineralocorticoid and glucocorticoid synthesis (Nakamura et al., 1966). The increase in total protein content suggests the development of adrenal hypertrophy (probably due to the activation of cellular mitotic activity), which was needed to support the adrenal gland hyperfunction secondary to stress from the increasing population size.

Other manifestations of the stress reaction include hypocellularity of the lymphoid tissue, the spleen in particular (Dominguez-Gerpe & Rcy-Mcmlez, 2001; Satoh et al., 2006), and our study also showed a decreased protein content and DNA/total protein ratio with increasing population size. Splenic hypoplasia is likely due to death and decreased mitotic activity of splenocytes. This is indicated by the accompanying change in other parameters. The observed decrease in GPI activity (the second key enzyme in anaerobic glycolysis) is an indication of trophic supplying the restriction of cells by glucose, which can lead to a reduction in their proliferative potential (Greiner et al., 1994). The inhibitory effect on carbohydrate metabolism in the lymphoid and bone marrow tissues is due to glucocorticoids hindering the capture and utilization of glucose by cells (Franchimont, 2004). The RNA/DNA ratio also increases due to increased chromatin transcriptional activity, and therefore, the prevalence of more differentiated cell forms (Schmidt & Schibler, 1995). The observed increase in the TBARS concentration along with the decreased catalase activity in splenocytes characterizes the development of a pro-/antioxidant (PO-AO) imbalance in rodents which occurred with increasing population size. The literature notes the possibility of the existence of specific imbalances in the PO-AO systems regulating cellular processes such as proliferation, differentiation, ageing, apoptosis, and cytolysis. The transition to a more pro-oxidant state can be a reason for the

Table 4. Univariate tests of significant differences for the biochemical parameters of *A. uralensis* trapped in the reference area and the EURT: the results of ANCOVA on the basis of the separate-slopes model.

			SS[b]			*F* (1.12)		*p*		Percentage changes of biochemical parameters of the EURT area[c]				
										Population abundance, ind./ 100 trap–day				
Parameter	Organ	(R^2)[a]	SS_{B1}	SS_{B2}	SS_R	F_{B1}	F_{B2}	p_{B1}	p_{B2}	10	20	30	40	50
Total lipids/protein ratio	Adrenals	0.62	52.5310	3.2268	32.4762	144.8	17.8	$<10^{-6}$	$<10^{-4}$	**−22**	**−29**	**−37**	**−47**	**−61**
TBARS concentration		0.51	68,563,38	597,596	107,013,20	55.1	9.6	$<10^{-6}$	0.002	**+48**	**+58**	**+66**	**+72**	**+78**
Total protein content		0.53	453,053.6	3733.4	289,458.1	139.0	2.3	$<10^{-6}$	0.131	**+65**	**+61**	**+58**	**+58**	**+58**
Total protein content	Spleen	0.71	41,823,90	87,537	25,987,83	144.0	6.0	$<10^{-6}$	0.015	**−15**	**−23**	**−32**	**−42**	**−53**
DNA/total protein ratio		0.72	0.004942	0.000114	0.002051	216.8	10.0	$<10^{-6}$	0.002	**−12**	**−18**	**−23**	**−30**	**−39**
Total RNA/DNA ratio		0.59	14.27387	0.31144	11.22435	114.4	5.0	$<10^{-6}$	0.026	**+43**	**+52**	**+59**	**+64**	**+68**
TBARS concentration		0.61	99,280,21	33,827	72,140,56	122.5	0.8	$<10^{-6}$	0.362	+59	**+111**	**+143**	**+163**	**+178**
Catalase activity		0.77	115,634.8	56.3	35,269.9	291.7	0.3	$<10^{-6}$	0.594	**−9**	**−18**	**−31**	**−45**	**−66**
GPI activity		0.81	40,228,97	14,821	861,204	418.0	3.1	$<10^{-6}$	0.081	−1.3	**−9**	**−20**	**−33**	**−53**

[a]The determination coefficient ($1 - SS_R/SS_t$) of the ANCOVA.
[b]The sum of squares on account: SS_{B1}: interaction effect of categorical (reference/impact) and continuous (population abundance) predictors. SS_{B2}: effect of categorical predictor; SS_R: errors of prediction.
[c]$\frac{(M_{EURT} - M_{reference})}{M_{reference}} \times 100\%$, where *M* is average value.
Bold font: biochemical changes from the EURT area are statistically significant at $p = 0.05$.

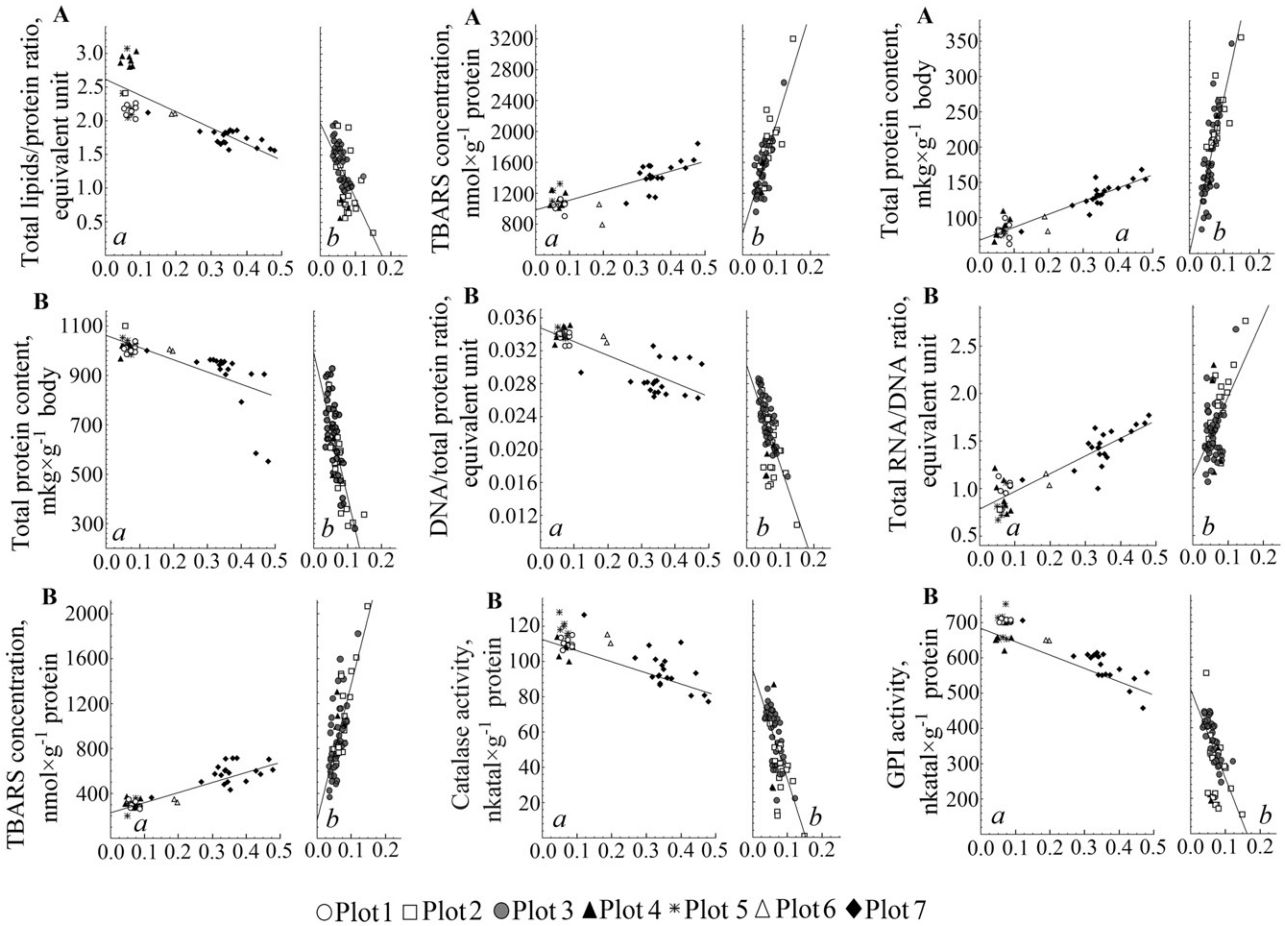

Figure 3. Effect of the whole-body radiation dose rate on biochemical parameters of two groups of *A. uralensis* within the EURT area according to the population size: from 2 to 26 ind./100 trap–day (a) and from 30 to 50 ind./100 trap–day (b). A – adrenal glands, B – spleen. X-axis: values of radiation dose rates, mGy/day.

suppression of the proliferative potential of the cells and, with a marked increase in the imbalance, can be the basis for the implementation of apoptosis and oxidative cytolysis (Lyu et al., 2007).

According to the investigations by Grigorkina and Pashnina (2007), the pygmy wood mice inhabiting the radioactively polluted biocenosis with a ^{90}Sr soil pollution density of 18.5 $MBq \times m^{-2}$ displayed multiple alterations in their haemapoietic systems, delays in the maturation of erythropoietic cells in the bone marrow, increases in the frequency of cells with micronuclei, and a general depression of immune system. In addition, the number of structural anomalies of

Table 5. Effect of whole-body radiation dose rate (x_1) and abundance of mice (x_2) on the biochemical parameters (y) in *A. uralensis* within the EURT area in two groups according to the population size (moderate, large): the results of multiple linear regression $y = b_0 \pm b_1 \times x_1 \pm b_2 \times x_2$.

		Population size							
		Moderate (from 2 to 26 ind./100 trap–day)				Large (from 30 to 50 ind./100 trap–day)			
		df(n − 2) = 41				df(n − 2) = 68			
Parameter	Organ	$b_0 \pm$ SE	$b_1 \pm$ SE*	β_1	$b_2 \pm$ SE*	β_2	$b_0 \pm$ SE	$b_1 \pm$ SE*	β_1	$b_2 \pm$ SE*	β_2
Total lipids/protein ratio, equivalent unit	Adrenals	**(26.7 ± 9.2) × 10^{-1}**	**(21.8 ± 3.8) × 10^{-1}**	−0.72	**(6.4 ± 7.0) × 10^{-3}**	−0.11	**(28.5 ± 1.8) × 10^{-1}**	**(76.7 ± 15.7) × 10^{-1}**	−0.42	**(31.0 ± 5.4) × 10^{-3}**	−0.49
TBARS concentration, nmol × g^{-1} protein		**(99.6 ± 5.5) × 10^{1}**	**(13.1 ± 2.2) × 10^{2}**	0.83	**(−1.6 ± 4.2) × 10^{-1}**	0.05	**(45.9 ± 1.5) × 10^{-1}**	**(134.5 ± 12.8) × 10^{2}**	0.77	**(78.0 ± 44.2) × 10^{-1}**	0.13
Total protein content, mkg × g^{-1} body		**67.3 ± 3.7**	**(1.8 ± 0.2) × 10^{2}**	0.92	**(7.2 ± 27.9) × 10^{-2}**	0.02	**20.0 ± 8.2**	**(19.4 ± 1.7) × 10^{2}**	0.73	**(245.0 ± 59.5) × 10^{-2}**	0.27
Total protein content, mkg × g^{-1} body	Spleen	**(10.0 ± 0.2) × 10^{2}**	**(7.4 ± 0.8) × 10^{2}**	−1.0	**6.7 ± 1.6**	−0.53	**(11.6 ± 0.7) × 10^{2}**	**(51.5 ± 6.3) × 10^{2}**	−0.66	**6.0 ± 2.2**	−0.23
DNA/total protein ratio, equivalent unit		**(36.8 ± 0.3) × 10^{-3}**	**(7.9 ± 1.2) × 10^{-3}**	−0.39	**(24.5 ± 2.2) × 10^{-5}**	−0.65	**(38.4 ± 1.5) × 10^{-3}**	**(73.4 ± 8.8) × 10^{-3}**	−0.50	**(29.1 ± 4.4) × 10^{-5}**	−0.49
Total RNA/DNA ratio, equivalent unit		**(72.1 ± 4.7) × 10^{-2}**	**1.6 ± 0.2**	0.76	**(7.4 ± 3.5) × 10^{-3}**	0.19	**(62.8 ± 2.0) × 10^{-2}**	**6.4 ± 1.7**	0.39	**(17.4 ± 5.9) × 10^{-3}**	0.31
TBARS concentration, nmol × g^{-1} protein		**184.4 ± 19.5**	**(6.9 ± 0.8) × 10^{2}**	0.67	**6.6 ± 1.5**	0.34	**143.8 ± 53.8**	**(107.8 ± 13.3) × 10^{2}**	0.67	**10.7 ± 4.5**	0.20
Catalase activity, nkatal × g^{-1} protein		**120.8 ± 2.6**	**57.5 ± 10.5**	−0.68	**(28.8 ± 19.6) × 10^{-2}**	−0.18	**138.3 ± 7.3**	**443.4 ± 63.1**	−0.50	**(153.6 ± 21.5) × 10^{-2}**	−0.51
GPI activity, nkatal × g^{-1} protein		**724.9 ± 10.3**	**(38.3 ± 4.4) × 10^{-1}**	−0.84	**(6.8 ± 7.9) × 10^{-1}**	0.08	**807.8 ± 21.6**	**(129.3 ± 18.8) × 10^{-1}**	−0.32	**(105.6 ± 6.4) × 10^{-1}**	−0.76

β (beta): standardized regression coefficient; df: degrees of freedom. *The values of the regression coefficients are given by modulus.
Bold font: *t*-values for regression coefficient ≥ critical $t_{0.05}$-value.

leukocytes increased, and there were signs of abnormal mitosis as well as some indications of the hemopoietic process being more tensions. A characteristic feature was a reduction in the spleen index in the background increase the relative weight of the adrenal glands (Grigorkina et al., 2008). However, these studies were not conducted to take into account the population size. It is not possible to verify the radiation stress or the action of stress factors independent of radiation exposure.

The absolute values of the b_1 coefficient of the linear equation $y = b_0 \pm b_1 \times x$ in the sample from the contaminated area were always higher (Table 3). This was evidenced by the greater effect of the influence factor "population abundance" on the neuroendocrine and hematopoietic systems of impacted animals. Because of this, the level of biochemical differences between the impact and reference samples depended on the abundance degree of the mice in the trapping plots (Table 4).

The trapping plots within the EURT area: effects of population size and radiation dose on biochemical parameters

It has previously been shown that metabolic reactions in tissues (blood plasma, erythrocytes, liver, myocardium, spleen, and adrenal glands) secondary to an increase in population size from 7.6 to 28.1 ind./100 trap–day in the EURT territory, which has a higher density of ^{90}Sr soil pollution (18.5 MBq × m^{-2}), are different from those within the reference area. These changes involve an increased level of oxidative metabolism, the inhibition of protein biosynthesis, evidence of cell hyperfunction, and the depletion of energy resources (Orekhova & Rasina, 2015). However, these studies of metabolic homeostasis were not conducted to take into account the whole-body radiation dose rate.

Our work shows that the functional-metabolic response in the spleen and adrenal glands of *A. uralensis* from the EURT area to the radiation burden (whole-body radiation dose rate) was modified by the population size (Figure 3; Table 5). The radiation effects were more pronounced when the population size was greater than 30 ind./100 trap–day. This raises the question of how much "additional" stress, caused by chronic radiation exposure, would have a negative effect on an organism living in an area with a large population. In this case, increased competition between individuals for environmental resources occurred, and, as a consequence, the stress response level increased. These animals therefore simultaneously incurred the powerful influence of radioactive and non-radioactive stressors. After all, the founder of the concept of stress, Selye (1975), distinguished harmful (distress) and useful (eustress) stress, the latter of which has an adaptive nature. The difference depends on the quantitative characteristics of exposure, that is, metabolic changes (distress/eustress) depend on the degree of influence exercised by the ecological factors.

In the ICRP Publication (ICRP, 2008) for the purposes of the radiation safety of biota, the ICRP introduces the concept of Reference Animals and Plants. Based on data from the biological effects of radiation for each reference organism, the

Derived Consideration Reference Levels (DCRLs) were defined. For the existing or planned exposure situation, doses of reference organisms are to be compared with relevant DCRLs. For the purposes of environmental protection, ICRP recommends the representative organism be the same as the actual object of protection under consideration. Each DCRL is regarded as a range of dose rate at which there is a possibility of harmful influences from ionizing radiation on the representatives of this type of reference animal or plant. Murine rodents may be chosen as the representative organisms for the EURT. The closest reference organism to these animals is the reference rat, for which the DCRL is 0.1–1 mGy/day.

In Group *b* (from 30 to 50 ind./100 trap–day), even the 95th percentile of the whole-body radiation dose rate was below the DCRL (25–75th percentiles were 0.051–0.078 mGy/day, with a median of 0.063 mGy/day, mean of 0.065 mGy/day, and maximum value of 0.149 mGy/day). On the basis of multiple regression equations (Table 5) the stress response can be compared when a moderate population size (Group *a*: from 2 to 26 ind./100 trap–day) to stress during the simultaneous action of high abundance of mice and the radiation burden. At a population size of 32 ind./100 trap–day (the median value) and a dose rate of 0.063 mGy/day, the stress response was similar to that from an individual from Group *a* in a small population size (10 ind./100 trap–day) with a radiation burden of 0.5–1 mGy/day, which exceeded the DCRL for three among the nine biochemical parameters (Table 6). The value of 0.1 mGy/day for mice trapped at a population level of 30 ind./100 trap–day is equivalent to a radiation burden of 0.7–1.3 mGy/day. With a population level of 40 ind./100 trap–day, it is also equivalent to a radiation burden of 0.8–1.5 mGy/day, and at 50 ind./100 trap–day a dose of 0.1 mGy/day is equivalent to a radiation burden of 0.9–1.7 mGy/day. The TBARS concentration, catalase activity, and DNA/protein ratio in the spleen and the total protein content in the adrenal glands were most sensitive to the interaction of factors. Thus, the functional-metabolic effects caused by a radiation burden of 0.1 mGy/day are amplified approximately tenfold with the simultaneous action of a large population (over 30 ind./100 trap–day) as an environmental stressor. When there is a large population size, within the ICRP concept of a radiological protection system, the optimization of protection of EURT biota should be aimed at reducing exposure to levels that are below the lower boundary of DCRL (less than 0.1 mGy/day). Otherwise, there will likely be harmful effects from the interactions of non-radiation and radiation factors, and a transition from adaptation (eustress) to dysadaptation (distress). The destructive effect of adrenocortical activity on the lymphatic system may lead to a prolonged disruption of the immune response and infectious complications.

Multivariate comparison of rodent samples: mathematical simulation for evaluating radiation as an environmental stressor. Role of environmental factors in the interpretation of radioactive effects on animal populations

The physiological and functional-metabolic states of animals within the EURT demonstrate the consequences of radiation accidents and, at the same time, are a result of the combined influence exercised by natural and anthropogenic factors on the individual and on the population as a whole. The linear dependence of the biochemical parameters (y) on the whole-body radiation dose rate (x_1) and population abundance (x_2) allows extrapolation of the data in point when x_1, $x_2 = 0$. This calculation will allow for optimal inter-group comparisons

Table 6. The extrapolation of the data from Group *b* (large population size: from 30 to 50 ind./100 trap–day) to Group *a* (moderate population size: from 2 to 26 ind./100 trap–day) on the basis of the effect of whole-body radiation dose rate (x_1) and abundance of mice (x_2) on the biochemical parameters(y) in *A. uralensis* within the EURT area according to the equation $y = b_0 \pm b_1 \times x_1 \pm b_2 \times x_2$*.

			The calculated y-values in the Group *b* at user-defined x_1- and x_2-values				The calculated x_1-values in the Group *a* at user-defined x_2-values and calculated y-values from the Group *b*							
			x_2, ind./100 trap–day											
							$x_2 = 10$				$x_2 = 20$			
Parameter	Organ	x_1, mGy/day	$x_2 = 32$ (a)	$x_2 = 30$ (b)	$x_2 = 40$ (c)	$x_2 = 50$ (d)	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
Total lipids/protein ratio, equivalent unit	Adrenals	0.063	1.38				0.59				0.59			
		0.1		1.16	0.85	0.54		0.69	0.84	0.98		0.69	0.84	0.98
TBARS concentration, nmol × g^{-1} protein		0.063	1556.9				0.42				0.18			
		0.1		2039.2	2117.2	2195.2		0.79	0.85	0.91		0.55	0.61	0.67
Total protein content, mkg × g^{-1} body		0.063	220.7				0.84				0.84			
		0.1		287.6	312.1	336.6		1.20	1.34	1.47		1.20	1.34	1.47
Total protein content, mkg × g^{-1} body	Spleen	0.063	645.7				0.39				0.30			
		0.1		467.1	407.1	347.2		0.63	0.72	0.80		0.54	0.62	0.71
DNA/total protein ratio, equivalent unit		0.063	0.023				1.36				1.05			
		0.1		0.021	0.018	0.015		1.70	2.06	2.43		1.39	1.76	2.12
Total RNA/DNA ratio, equivalent unit		0.063	1.58				0.49				0.45			
		0.1		1.78	1.96	2.13		0.62	0.73	0.83		0.57	0.68	0.79
TBARS concentration, nmol × g^{-1} protein		0.063	1166.3				1.33				1.23			
		0.1		1543.5	1650.9	1758.3		1.88	2.03	2.18		1.78	1.94	2.09
Catalase activity, nkatal × g^{-1} protein		0.063	61.3				1.03				1.03			
		0.1		47.9	32.6	17.2		1.27	1.53	1.80		1.27	1.53	1.80
GPI activity, nkatal × g^{-1} protein		0.063	388.4				0.88				0.88			
		0.1		361.7	256.1	150.6		0.95	1.22	1.50		0.95	1.22	1.50

*For the regression coefficients, see Table 5.

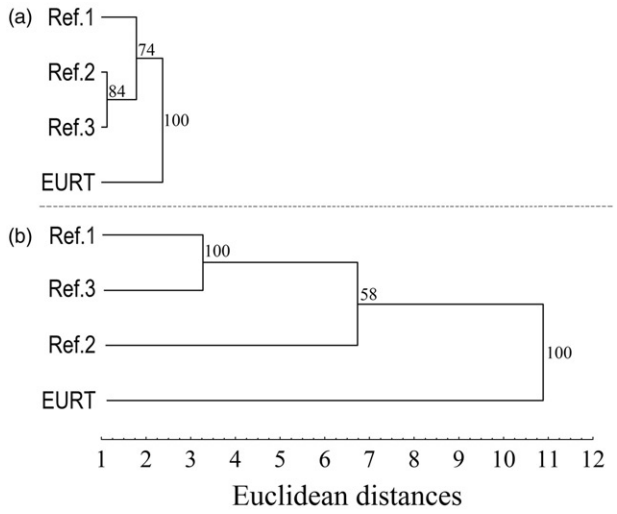

Figure 4. Similarity measure for four rodent samples: the results of cluster analysis on the basis of nine biochemical parameters; a – median (extra) values, b – median (exp) values. The numbers indicate the bootstrap support based on 1000 bootstrap pseudoreplicates of a data matrix (Supplement 3).

after the elimination of the x_1-, x_2-effects. The y_i-value, extrapolated to the x_1, $x_2 = 0$, is defined by the formula y_i (extra) $= y_i \pm b_1 \times x_{1i} \pm b_2 \times x_{2i}$, where yi is the experimentally (exp) obtained value of the biochemical parameter for each (*i*) animal in the condition, where x_{1i} = values of radiation dose and x_{2i} = values of population abundance. It is obvious that the mean value for y_i(extra)-values is approximately equal to the b_0-value.

The multivariate comparison of rodent samples is presented in Figure 4 received for nine parameters, where Figure 4(a) is based on the median (extra) values and Figure 4(b) is based on the median (exp) values. As shown in Figure 4(a), every internal branch with a bootstrap proportion of >70% defined a true clade in this simulation. The cladogram essentially does not change, if be used another similarity measure (Supplement 4). Based on these cladograms, it can be concluded that the greatest similarity showed samples from areas of Ref. 2 and Ref. 3, in which the animals were caught in August. The greatest similarity can be related to the geographical proximity of areas (Figure 1) which did not manifest itself when was the x_2-effect (Figure 4(b)). The metabolic features of rodents from the Ref. 1 area may be linked to when they were caught in the October. This cannot be excluded, especially since significant geographical distances exist between the sites of Refs. 2–3 and Ref. 1 (Figure 1). Lastly, Figure 4 showed the greatest originality of the EURT samples. Unfortunately, we cannot give a clear "ecological" interpretation of the isolated samples of the EURT from all reference groups in this simulation despite the elimination of the x_1-, x_2-effects. The EURT could simply be another study area. Its uniqueness may be related to the removal of any anthropogenic influence on the biota as a result of the termination of human economic activity within this area. On the other hand, despite the geographical proximity, the EURT and Ref. 1 samples could fall into different clades because of different times of trapping (EURT – July, Ref. 1 – October). Regardless, extrapolation of the data leads to a significant decrease in the biochemical differences between the EURT area and reference samples after the elimination of the x_1-, x_2-effects (compare Figure 4(a,b)).

Figure 4(b) shows that internal branches are explained by population abundance and/or radiation exposure. The united clade of Ref. 1 and Ref. 3 is due to the similarity in the population level values (28 ind./100 trap–day and 21 ind./100 trap–day, respectively) leading to a similar status of the neuroendocrine and hematopoietic systems. The Ref. 2 area obtained very poor support by bootstrap analysis (59%), which suggests a low probability of a corresponding clade in this simulation. When changing similarity measures, we can get a united clade from the EURT and Ref. 2 samples (Supplement 4). These samples are characterized by an elevation in function of the stress-realizing system of the animals. In the Ref. 2 area, this was caused by the high population size at the site of capture (65 ind./100 trap–day), and in the EURT zone this was caused by the radiation dose (as the median value of population size was 30 ind./100 trap–day).

Therefore, an account of environmental factors such as rodent population number, season, year of capture, and geographical distances allowed us to correct the results of radiation effects and their interpretation.

Conclusion

The presence of overpopulation in both the reference plot and the EURT zone in areas with ^{90}Sr contamination densities of 3.3–22.3 $MBq \times m^{-2}$ caused functional-metabolic changes in the spleen and adrenal glands of *A. uralensis*, corresponding to a nonspecific adaptive stress reaction. This was expressed in the delipidisation of the adrenocortical cells, increased levels of LPO as the main mechanism for steroidogenesis, growth of the protein components of the adrenal glands to maintain their hyperfunction, as well as immunosuppression associated with the restriction of carbohydrate providence of splenocytes, reduction in them of DNA synthesis, and the development of a pro-/antioxidant imbalance.

The reactivity of the neuroendocrine and hematopoietic systems of animals in areas of large population sizes was higher in the EURT zone compared with the reference group. This is explained by additional stress due to the influence of chronic irradiation. The radiation dose biochemical effect was modified by the factor of "relative abundance of mice". The functional-metabolic effects caused by the radiation burden of 0.1 mGy/day were amplified approximately 10 times by the simultaneous presence of a large population (over 30 ind./100 trap–day) as an environmental stressor. When there is a large population size, within the ICRP concept of radiological protection, optimizing protection of EURT biota should be aimed at reducing exposure levels to below the lower boundary of the DCRL (less than 0.1 mGy/day). Otherwise, we should expect a possibility of a harmful effects resulting from interactions of non-radiation and radiation factors, and a transition from adaptation (eustress) to dysadaptation (distress). The destructive effect of adrenocortical activity on the lymphatic system may lead to a prolonged disruption of the immune response and infectious complications.

Disclosure statement

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