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ADAPTIVE RESPONSE IN FROGS CHRONICALLY EXPOSED TO LOW DOSES OF IONIZING RADIATION IN THE ENVIRONMENT

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ABSTRACT

Using the micronucleus assay, decreased DNA damage was found to be induced by high doses of ionizing radiation in hepatic cells from leopard frogs inhabiting a natural environment with above-background levels of ionizing radiation, compared to cells from frogs inhabiting background areas. The data obtained from a small number of animals suggest that stress present in the above-background environment could induce an adaptive response to ionizing radiation. This study did not reveal harmful effects of exposure to low levels of radioactivity. On the contrary, stress present in the above-background area may serve to enhance cellular defence mechanisms.

I. INTRODUCTION

Ionizing radiation can induce DNA breaks in cells. These breaks may lead to improper segregation of DNA fragments into the two daughter nuclei at cell division; the fragments may subsequently be packaged into micronuclei. Micronuclei are fragments or whole chromosomes that were not incorporated into the main cell nucleus during cell division [1]. The frequency of micronucleus formation in cells that have been exposed to high doses of radiation and allowed to repair, therefore, represents a measure of residual DNA damage following radiation exposure.

Environmental radiation doses and dose rates are generally well below those expected to produce mortality or significant measurable detrimental effects. Although environmental radiation exposures may modulate the frequency of micronucleus formation, a multitude of other environmental and biological factors will contribute to inter-individual variability. Therefore, attempting to establish a relationship between exposure and effect with low signal-to-noise ratios, can translate into the need to gather a large number of measurements. To reduce the number of animals required, and as higher signal-to-noise ratios are expected at higher radiation doses, measurements of the frequency of micronuclei in sampled biota subsequently exposed to a high dose of ionizing radiation was considered. If above-background radiation affects the response to subsequent high radiation doses, measurements of micronucleus frequency could be used to look for an adaptive response.

The adaptive response is defined as the induction of resistance to a stressor, including high doses of ionizing radiation, by prior exposure to a small "adapting" stress. Chronic low doses (such as typical environmental exposures) can be considered an "adapting" stress. The adaptation phenomenon is not radiation-specific, but is an example of a more general stress response [2]. Cross-adaptation has been observed between radiation and metals [3], chemicals [4, 5] and hyperthermia [6, 7].

The objective of this preliminary work was to evaluate the feasibility of using the micronucleus assay for the detection of an adaptive response triggered by chronic exposure to environmental stressors (including low levels of ionizing radiation). If measurable, such induced competence could provide a biomarker for monitoring biological effects in natural populations.

Leopard frogs (*Rana pipiens*) were chosen for this study as they are common across North America, utilize both terrestrial and aquatic environments and could be used as an indicator species for environmental assessments.

II. MATERIALS AND METHODS

II.A. Frog habitats

Hepatic cells were obtained from leopard frogs captured in Duke Swamp, an area with above-background tritium and carbon-14 levels, and from two background areas (Dew Drop Lake and Twin Lake). These areas are all located on the Chalk River Laboratory property (Figure 1).

The tritium levels measured in Dew Drop Lake and Twin Lake in 2003 were about 475 and 707 Bq/L, respectively. The estimated natural background radiation is about 2.4 mGy/year. Within Duke Swamp, the frogs were captured in an area where the tritium porewater concentrations ranged between 5,000 and 35,000 Bq/kg. The carbon-14 concentrations in mosses were estimated to be between 237 and 1,000 Bq/kg C (based on a study conducted in 2001). In the Duke Swamp sampling area, the estimated dose to biota was 1 mGy/year above-background.

II.B. Frog handling

The frogs were captured using a dip net. The captured frogs were placed in a plastic container containing icy water and equipped with a perforated cap. The frogs were kept on ice for a maximum of 2 h. The frogs were then sacrificed by percussion stunning followed by decapitation.

II.C. Cell culturing

The livers of adult leopard frogs, *Rana pipiens*, were carefully removed and placed inside a sterile culture plate. The livers were covered with sterile phosphate-buffered saline solution (PBS) containing Gentamycin (to reduce bacterial contamination). The PBS solution (5 L) was prepared using 40 g NaCl, 1 g KCl, 8.6g $Na_2HPO_4 \cdot 7H_2O$ and 1 g KH_2PO_4 in deionized, distilled, filter-sterilized water. The pH was adjusted to 7.4 using dilute HCl and the volume was brought up to 5 L. The PBS was filter-sterilized and was supplemented with a gentamycin solution: 5.5 mL of gentamycin (Invitrogen Canada Ltd., Burlington ON) per 550 mL of PBS solution.

The extracted livers were cut into very small pieces (less than 1 mm^3) using scalpels. The process of physical separation released free hepatocytes from the liver, although some tissue pieces were still visible. The cell suspension was transferred to two 15-mL polypropylene centrifuge tubes, and centrifuged at 125 x *g* for 2 min. Most of the supernatant was removed, bringing the volume of the pellet and PBS solution to about 1 mL. The pellet was resuspended in 4 mL of PBS, and 100 µL of collagenase solution (1000 units/mL; Worthington Biochemical Corp., Freehold NJ) was added per 1.0 mL of cell suspension. The suspension was gently inverted for 10 min.

The cell suspension was centrifuged once again (125 x *g* for 2 min), and virtually all the supernatant was removed, leaving only the cell and tissue pellet at the bottom of the tube. The pellet was resuspended in 5 mL culture medium, consisting of 50% Modified L-15 Leibovitz Culture Medium (Sigma-Aldrich Canada Ltd., Oakville, ON), 39% deionized, distilled, filter-sterilized water, 10% Fetal Calf Serum (Invitrogen Canada Ltd.) and 1% gentamycin solution, and 1.25 mL of the resulting cell suspension ($1x10^7$ to $1x10^8$ cells/mL) was transferred to four T-25 culture flasks. The cultures were then placed at an angle and incubated at room temperature.

II.D. Experimental treatments

For the laboratory (*in vitro*) experiments, the cells were maintained in culture for approximately 24 h prior to being irradiated. Some samples were subjected to an "adapting" irradiation dose (100 mGy) delivered at a dose rate of about 5 mGy/min (GammaBeam 150C ^{60}Co irradiator, Atomic Energy of Canada Limited). The cultures were then incubated at room temperature for 3 h, after which some samples received a 4 Gy "challenge" dose (at a dose rate of about 15.6 Gy/min, Gammacell 220, Atomic Energy of Canada Limited (AECL)). Apart from the actual exposures to ionizing radiation, care was taken for all samples to be subjected to the same treatments (including transport and handling).

For the field (*in vivo*) experiments, the frogs were removed from the icy water and placed into a small plastic container equipped with a perforated cap. The frogs received a "challenge" dose of 4 Gy (at a dose rate of about 15.6 Gy/min, Gammacell 220, AECL), and were placed back into the icy water. The frogs were sacrificed minutes after this

radiation exposure. Primary hepatic cell cultures were then established and maintained in cell cultures for about 24 h.

In preparation for the micronucleus assay, 10 µL of Cytochalasin B (0.2 mg/ml) (Sigma-Aldrich Canada Ltd.) was added per 1.0 mL of culture suspension to arrest cells at cytokinesis, 48 h before the cells were harvested and fixed onto slides.

II.E. Cell harvest and slide preparation

The cultures were gently aspirated to dislodge the cells from the culture flask walls. The suspension was then transferred to a 15-mL polypropylene centrifuge tube and the cultures were centrifuged at 150 x *g* for 8 min at 7°C. Most of the supernatant was removed and the pellet was gently resuspended in the remaining supernatant.

Soft fixative solution (0.75 mL of 10% acetic acid) was added to the cells. After 5 min, 0.75 mL of fixative solution (3:1 methanol/acetic acid containing 10% formaldehyde) was also added. Five minutes later, the cells were centrifuged (150 x *g* for 8 min at 7°C). Most of the supernatant was removed and the pellet was resuspended in 1.5 mL of fixative solution (3:1 methanol/acetic acid). Following a standing time of 5 min, the cells were centrifuged again (150 x *g* for 8 min at 7°C) and the supernatant was discarded. Typically, a volume of fixative corresponding to twice the volume of the pellet was added to the tube. The slides were immediately prepared from the fixed cells. The remaining fixed cells were stored at -20°C.

Acid-washed glass slides, stored in 95% ethanol at -20°C until use, were dipped a few times into an ice-water bath to remove the ethanol. The slides were dried, and 12 µL of the cell suspension were dropped onto each slide. The slides were flushed three times with fixative (3:1 methanol/acetic acid) and then dried over a hot water bath (80°C). Slides were stained for 30 to 60 s in acridine orange dye (50 µg/mL; BDH Inc., Toronto ON), then rinsed twice with deionized, distilled water. A rectangular cover slip was placed over the cells. As acridine orange is sensitive to light, the slides were stored in the dark at room temperature until evaluation.

II.F. Micronucleus Assay

Blinded hepatocyte slides were scored under a fluorescence microscope (fitted with a FITC filter cube) at 400x magnification, according to the criteria described in Fenech *et al.* (2003) [1]. One thousand binucleate cells (BNC) were scored per treatment for the presence of micronuclei (MN).

The data were reported as the MN frequency (number of MN per 100 BNC) as calculated using equation 1:

$$MNfrequency(MN/100BNC) = \frac{((\#BNCwith1MN) + 2(\#BNCwith2MN) + 3(\#BNCwith3MN))}{\#BNC} \quad \text{EQ.1}$$

The standard deviation of the MN frequency was calculated using equation 2:

$$STD = \sqrt{\frac{(\#BNC + \#MN)}{(\#BNC \bullet \#MN)}} \bullet \frac{\#MN}{\#BNC} \quad \text{EQ.2}$$

III. RESULTS

Both laboratory (*in vitro*) and field (*in vivo*) experiments were conducted. The *in vitro* experiments were conducted using hepatic primary cell cultures established from the livers of frogs collected in areas with either background or above-background levels of ionizing radiation. In these *in vitro* experiments, the 100 mGy "adaptive" and the 4 Gy "challenge" doses were delivered *in vitro*. The field experiments compared the micronucleus frequency in hepatocytes obtained from frogs collected in an area with above-background levels of ionizing radiation and frogs collected in background areas, after *in vivo* 4-Gy gamma-irradiation.

III.A. In Vitro Experiments

Two female leopard frogs and one male leopard frog were used in this *in vitro* study (Table 1). Based on their sizes, the two female frogs were likely at least a year old; the male frog, which was smaller (52 mm), may have been younger. The MN frequency in primary hepatocyte cultures was determined after the cells had been exposed to different doses of ionizing radiation (Table 2). Low "adaptive" doses of 0 or 100 mGy ^{60}Co-gamma radiation were delivered, followed 3 h later by exposure to 0 or 4 Gy ^{60}Co-gamma radiation.

During the summer of 2003, *in vitro* experiments were conducted on hepatocytes from female frogs captured in Dew Drop Lake (Experiment 1) and Duke Swamp (Experiment 2). In the fall of 2004, an experiment was conducted on hepatocytes from a male frog captured in Twin Lake (Experiment 1A). The MN data are summarized in Table 3 and illustrated in Figure 2.

The results for the female frog from Dew Drop Lake showed that MN frequencies for hepatocytes exposed *in vitro* to 0 and 100 mGy are not likely to be significantly different, since the values differ by less than ± 2 standard deviations. Hepatocytes from this frog had higher MN frequencies when exposed to only the 4 Gy "challenge" dose (differing by more than 2 standard deviations). The MN frequencies in cells pre-exposed to a 100 mGy "adaptive" dose followed by the 4 Gy exposure were lower than the MN

frequency in cells exposed to the 4 Gy dose alone (differing by more than 2 standard deviations). Similar results were obtained in Experiment 1A.

Similary, the MN frequencies in hepatocytes obtained from the frog captured in Duke Swamp (an area with above-background levels of ionizing radiation) and then exposed to 0 and 100 mGy *in vitro* were not likely to be significantly different. However, exposure of these hepatocytes to 4 Gy radiation did not greatly affect the MN frequency, with or without prior "adapting" exposure to 100 mGy.

III.B Field experiments

For the preliminary field experiments, the hepatocytes from six leopard frogs, exposed to a 4-Gy "challenge" dose *in vivo*, were used (Table 4). One frog, a female, was collected from Dew Drop Lake. Two more frogs (one male and one female) were captured from Twin Lake. In addition, three female frogs were captured in Duke Swamp. Based on their sizes, all frogs were likely at least a year old.

MN frequencies were determined for all six frogs (Table 5). Frogs collected from the background areas tended to have higher MN frequencies than frogs collected from Duke Swamp, where background radiation was higher. Background area MN frequencies fell within +/- 2 standard deviations of each other, as did above-background area MN frequencies; however, the MN frequencies for background radiation areas were all different by more than 2 standard deviations from the MN frequencies for above-background areas (Figure 3), suggesting that there would be a significant difference.

IV. DISCUSSION

IV.A In vitro Experiments

The micronucleus frequency in primary hepatocyte cultures derived from frogs collected in areas with background radiation levels (Twin Lake and Dew Drop Lake) was increased by exposure to 4 Gy ionizing radiation. This increase was reduced if the cells were exposed to 100 mGy ionizing radiation before the high dose exposure, although the same 100 mGy exposure had no effect on micronucleus frequency when the cells were not exposed to the 4 Gy "challenge" dose.

However, 4 Gy ionizing radiation exposure had no effect on the micronucleus frequency in primary hepatocyte cultures derived from frogs collected in Duke Swamp, an environment known to have above-background ionizing radiation levels. None of the radiation treatments delivered *in vitro* had any marked effect on micronucleus frequency in these cells.

IV.B Field Experiments

A lower micronucleus frequency was observed in cells from frogs from the area with above-background levels of ionizing radiation, compared with frogs from background areas, following an *in vivo* exposure to a high dose.

IV.C Conclusions

The preliminary data described here give no indication that frogs are harmed by chronic low-level environmental ionizing radiation exposure. However, a decreased sensitivity to radiation damage (as measured using the micronucleus assay) was seen in hepatocytes from frogs collected in Duke Swamp, whether the radiation dose was delivered to live frogs or cultured cells. This suggests that some stressor in Duke Swamp was acting to induce protection of DNA from the effects of high dose radiation exposure; perhaps the elevated radiation levels, although other environmental factors may also be responsible. This induced resistance may provide a biomarker for biological effects monitoring in natural populations; however, more work is needed to establish whether the environmental exposure to ionizing radiation is indeed the stressor responsible for the observed reduction in micronucleus frequency.

Future work will address the natural variation in micronucleus induction in frog populations, as only a small number of frogs from a small number of environments have been tested so far.

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Table 1: *In vitro* Experiments: Origin of the Primary Liver Cells Cultures

Experiment #	Area of frog capture	Date of frog capture	Gender of the frog	Length from nose to fork
1	Dew Drop Lake (B)	2003 July 12	Female	65 mm
1A	Twin Lake (B)	2004 October 5	Male	52 mm
2	Duke Swamp (C)	2003 July 24	Female	77 mm

B: Background;
C: Above-background ionizing radiation levels.

Table 2: *In vitro* Experiments: Summary of the Irradiation Treatments

Experiment #	Adaptive Dose (mGy)	Time Lapse Between Irradiations (h)	Challenge Dose (Gy)
1	0	3	0
1	100	3	0
1	0	3	4
1	100	3	4
1A	0	3	0
1A	100	3	0
1A	0	3	4
1A	100	3	4
2	0	3	0
2	100	3	0
2	0	3	4
2	100	3	4

Table 3: *In vitro* Experiments: Scoring Data

Exp. #	Irradiation Treatment (adaptive, challenge)	# Cells	# BNC	# BNC with at least 1 MN	# BNC with at least 2 MN	# BNC with 3 or more MN	MN frequency (MN/100 BNC)
1	0 mGy, 0 Gy	13499	1002	50	11	0	7.2
1	100 mGy, 0Gy	14548	1003	46	8	1	6.5
1	0 mGy, 4 Gy	13859	1010	98	22	3	15.0
1	100 mGy, 4Gy	15140	1032	61	14	3	9.5
1A	0 mGy, 0 Gy	23114	1001	41	8	1	6.0
1A	100 mGy, 0Gy	26766	1000	48	6	0	6.0
1A	0 mGy, 4 Gy	20592	1004	121	18	2	16.2
1A	100 mGy, 4Gy	28609	1007	61	8	2	8.2
2	0 mGy, 0 Gy	12119	1002	42	6	0	5.4
2	100 mGy, 0Gy	13395	1001	45	7	1	6.2
2	0 mGy, 4 Gy	11990	1002	51	7	1	6.8
2	100 mGy, 4Gy	11273	1001	46	8	1	6.5

Exp = Experiment; BNC = Binucleate cell(s); MN = miconucle(us)/(i).

Table 4: Field Experiments: Frog Information

ID Number	Area of frog capture	Date of capture	Gender	Length (nose to fork)
B-2	Twin Lake (B)	2003 July 08	Male	70 mm
B-3	Dew Drop Lake (B)	2003 July 23	Female	82 mm
B-4	Twin Lake (B)	2003 July 29	Female	77 mm
C-1	Duke Swamp (C)	2003 July 08	Female	73 mm
C-2	Duke Swamp (C)	2003 July 22	Female	71 mm
C-3	Duke Swamp (C)	2003 July 23	Female	81 mm

B: Background;
C: Above-background ionizing radiation levels.

Table 5: Field Experiments: Scoring Data

ID Number	# Cells	# BNC	# BNC with at least 1 MN	# BNC with at least 2 MN	# BNC with 3 or more MN	MN frequency (MN/100 BNC)
B-2 (MS)	10959	1004	101	16	4	14.4
B-3 (MS)	9095	1002	92	16	4	13.6
B-4 (MS)	9121	1018	101	18	3	14.3
C-1 (MS)	9510	1007	51	8	0	6.6
C-2 (MS)	10573	1018	58	10	0	7.7
C-3 (MS)	10529	1003	43	11	1	6.8

BNC = Binucleate cell(s); MN = miconucle(us)/(i).

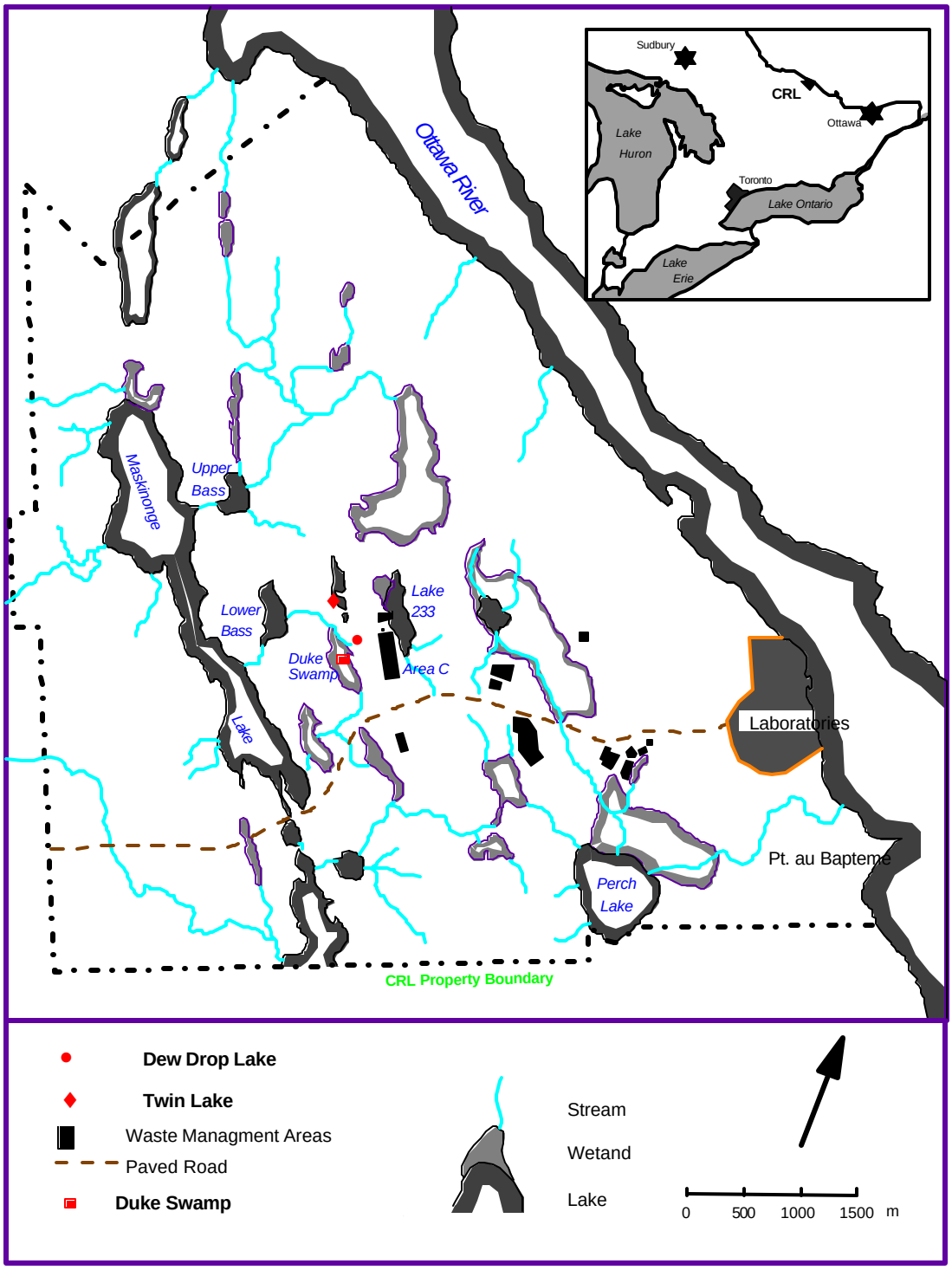

Figure 1: **Map of the Chalk River Laboratory Site.**
The sampling areas are shown in red.

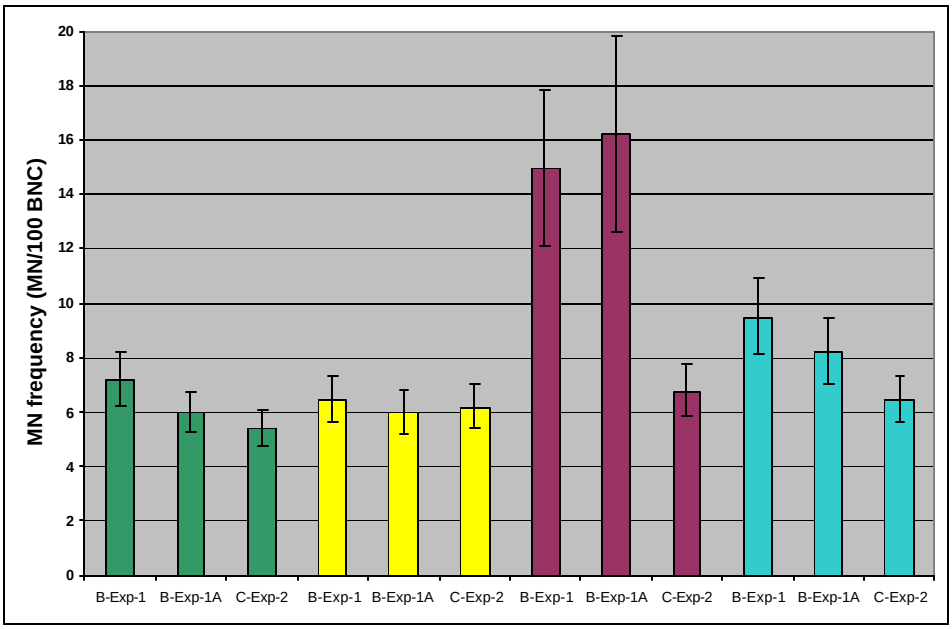

Figure 2: Preliminary Laboratory Experiments: Measure of the MN Frequency in *Rana pipiens* Hepatocytes irradiated *in vitro*.

The green bars represent 0 mGy (adaptive dose) and 0 Gy (challenge dose), the yellow bars 100 mGy (adaptive dose) and 0 Gy (challenge dose), the red bars 0 mGy (adaptive dose) 4 Gy (challenge dose), and the blue bars 100 mGy (adaptive dose) and 4Gy (challenge dose). The error bars illustrate two standard deviations.

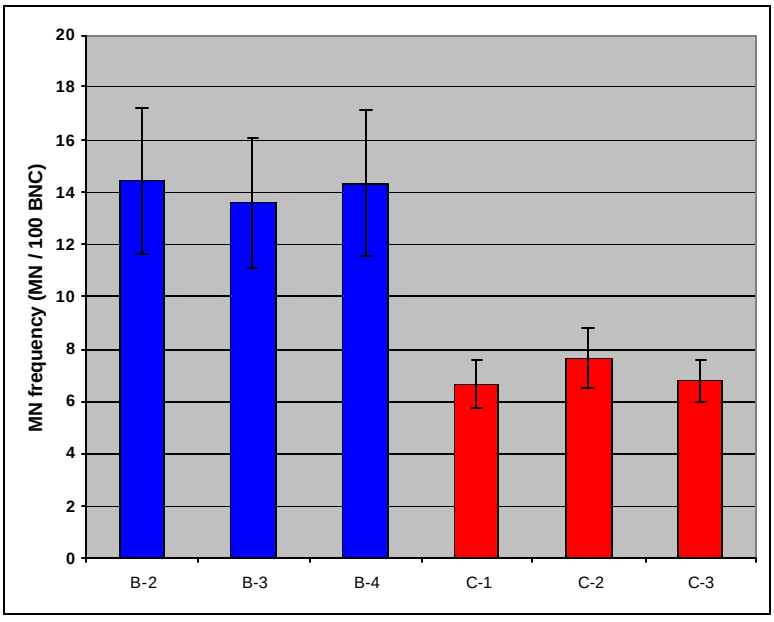

Figure 3: Preliminary Field Experiments: Measure of the MN Frequency in *Rana pipiens* Hepatocytes irradiated *in vivo*.

The blue bars represent frogs captured in the background areas and the red bars represent frogs collected in an area with above-background ionizing radiation levels. The error bars illustrate two standard deviations.