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Selected Morphological Changes in *Artemia franciscana* after Ionizing Radiation Exposure

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Abstract Nauplii of *Artemia franciscana* were irradiated by the doses of 0.25, 0.5, 1.0, and 2.5 kGy ^{60}Co. Dimensions of the body length, body width, intestine width, intestine epithelium width, and intestine lumen width, as well as the mutual ratios of dimensions were determined in 126 specimens. Ratios of the body length/body width (3.98, 3.60, 3.59, and 3.45 vs. 4.13 of control group), and ratios of the intestine epithelium width/intestine lumen width (0.64, 0.52, 0.51, and 0.45 vs. 0.85 of control group), according to the doses, were the most important parameters of evaluation of dependence of morphological changes on radiation doses.

Keywords *Artemia franciscana* · Gamma radiation · Morphological changes · Intestine epithelium

As physical and chemical tests alone are not sufficient enough for the assessment of potential effects on aquatic organisms, bioassays are required for the integrated evaluation of water pollution (Lu et al. 2011). Different kinds of invertebrates have been used since the 1950s for radiobiological research (Grosch and Erdman 1955). The important difference which distinguishes between animal tests and other tests, for example, in tissue cultures, is the complexity of live systems which cannot be simplified. If we use tissues and cells for research, then this is the study of partial systems, not a simplified organism. All restoration mechanisms which are available in the organism cannot fully operate in the partial system (Burlakova and Naidich 2006). The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes demands that the number of experiments on these animals should be minimized. The Three R's concept of Reduction, Refinement and Replacement defined according to the Council of Europe (1976) was adopted. The different configurations of the subacute biotest in *Artemia franciscana* (or *A. salina*) comply with the above mentioned requirements. *Artemia franciscana* Kellogg, 1906, belongs to the *Anostraca* order and it forms a separate group of *Crustacea* in the *Branchiopoda* class. *Crustacea* in this order are characterized by a fractional chitin exoskeleton. The hatching of organisms in dormant stages is advantageous, this means that a sufficient amount of individuals is formed. The above mentioned facts were used, for example, during observation and comparison of the cosmic radiation effects on the rest stages (Gaubin et al. 1986) or during the observation of the ^{60}Co gamma radiation effects and fast neutron effects (29 MeV) in *Artemia* dormant stages and their subsequent mortality and the changes after hatching (Iwasaki and Maruyama 1971). The cosmic radiation effect in *Artemia* rest stages was also investigated in the Biostack project tested during the Apollo 16 mission when a reduction of hatching from 90 % to 10 % was observed (Gaubin et al. 1990; Ruther et al. 1974; Planel et al. 1994). An increased mortality and various somatic deviations were also observed in nauplius

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larvae (Heinrich 1977). Shortening of extremities and abnormal formation of thoracic or abdominal parts were observed most frequently. The adult individuals did not achieve the standard size and ten times more malformations compared to the individuals in the control group were observed in the individuals in the next generation (Graul et al. 1975).

Our previous work was mainly focused on the observation of *A. franciscana* lethality depending on the ionizing radiation doses. The LD_{50} was determined 96 h after exposure to a ^{60}Co dose from 600 to 700 Gy (Dvořák and Beňová 2002). Due to the fact that *A. franciscana* underwent fast developmental stages (instar), it was possible to monitor the morphological changes in the organisms in a relatively short time interval depending on the doses exposed.

Materials and Methods

We used salt water with salinity of 47 g L^{-1} and pH value of 7.6 ± 0.1 in our experiments (Sterba 1983; Dvořák et al. 2010). Composition (g L^{-1}): 23.9 NaCl; 10.83 $MgCl_2 \cdot 6H_2O$; 2.25 $CaCl_2 \cdot 6H_2O$; 0.68 KCl; 9.06 $Na_2SO_4 \cdot 10H_2O$; 0.20 $NaHCO_3$; 0.04 $SrCl_2 \cdot 6H_2O$; 0.099 KBr; 0.027 H_3BO_3.

Hydration and hatching were carried out within 24 h at temperature of 25°C. The nauplius stages were divided after hatching into 6 plastic bottles each with a volume of 1 L. Due to retaining a sufficient amount of the air, only 250 mL of salt water solution with approximately 100 individuals were put into each bottle. Five bottles with the organisms were exposed to ^{60}Co gamma radiation with the doses of 0.25, 0.5, 1.0 and 2.5 kGy (dose rate of 1.875 kGy h^{-1}) within 40 h from the beginning of hydration of dormant stages. The sixth bottle was used as a control group.

After an exposure of ionizing radiation dose, the bottles with individuals were kept in a thermobox at a temperature of (20 ± 1)°C. The nauplius stages 48 h after exposure were moved from the bottle onto a glass slide. Devitalization and fixation of the individuals were done with 36 % formaldehyde solution. After coverage with the cover glass, the redundant fluid outside the cover glass was removed by filtration paper. Then, microphoto documentation was done by means of Olympus CX 31 Microscope and Olympus E 410 cameras. The sizes and the dimensions of the observed objects were always determined by means of Olympus QuickPhoto Micro 2.3 software. In total, thirty (30) individuals were monitored in the control group, with 33 individuals exposed to 0.25 kGy, 24 individuals exposed to a dose of 0.5 kGy, 28 individuals exposed to 1 kGy, and finally 11 individuals exposed to a dose of 2.5 kGy.

In the framework of the statistical data analysis, the arithmetic average of the monitored parameter (mean) and the standard error of mean (SEM) are always shown in figures and tables. Testing of the dependence of the monitored parameters (i.e. body dimensions, intestine dimensions and their mutual ratios) on the doses was carried out by the one-factor ANOVA analysis at the significance levels of $\alpha = 0.05$ and $\alpha = 0.01$.

Results and Discussion

The EU efforts to reduce the number of trial vertebrates can be realized by a substitution of conventional tests with alternative biotests. Unlike insulated tissue cultures the biotests in plankton animals represent a model based on a complex live organism. These are represented by the experiments which are more similar to natural conditions compared to the inbreeding lines of trial vertebrates.

Radiosensitivity of *A. franciscana* was approximately by two orders lower compared to mammals. This was given by the phylogenetic classification of the organism. Unlike the previous studies in which ^{60}Co gamma radiation effect in the *Artemia* dormant stages and the consecutive changes after hatching had been observed (Iwasaki and Maruyama 1971), our experiment was focused on radiation application within some hours after hatching.

The *A. franciscana* nauplius stages exposed to the doses of 2.5, 0.5, 1.0 and 2.5 kGy showed 48 h after exposure some differences in the morphological signs which were dependent on the doses.

A very fast and indirect development through the individual developmental stages (instars), which differ from each other, was found in the nauplius stages of *Artemia* genus. This is evident in Fig. 1a, b in which a fresh hatched individual compared to a 48 h-old individual in the control group is shown. The nauplius stages after hatching measured from 0.4 to 0.5 mm. Labrum which can move filtered food into the mouth was found on the ventral side of the head part. The second part, i.e. thoracoabdominal part, was non-segmented in nauplius larva of the first instar, see Fig. 1a. This part was remarkably and relative segmented during development, see Fig. 1b, and appendages were developed (Weekers et al. 2002).

The shortening of the extremities and the abnormal formation of thoracic and/or abdominal parts were observed in the nauplius stages which were obtained from dormant stages exposed to the cosmic radiation. The adults did not achieve the standard size and ten times more malformations were observed in the individuals of the next generation compared to the control group (Heinrich 1977; Graul et al. 1975). The shortening of the body length and the absence of development of thoracoabdominal

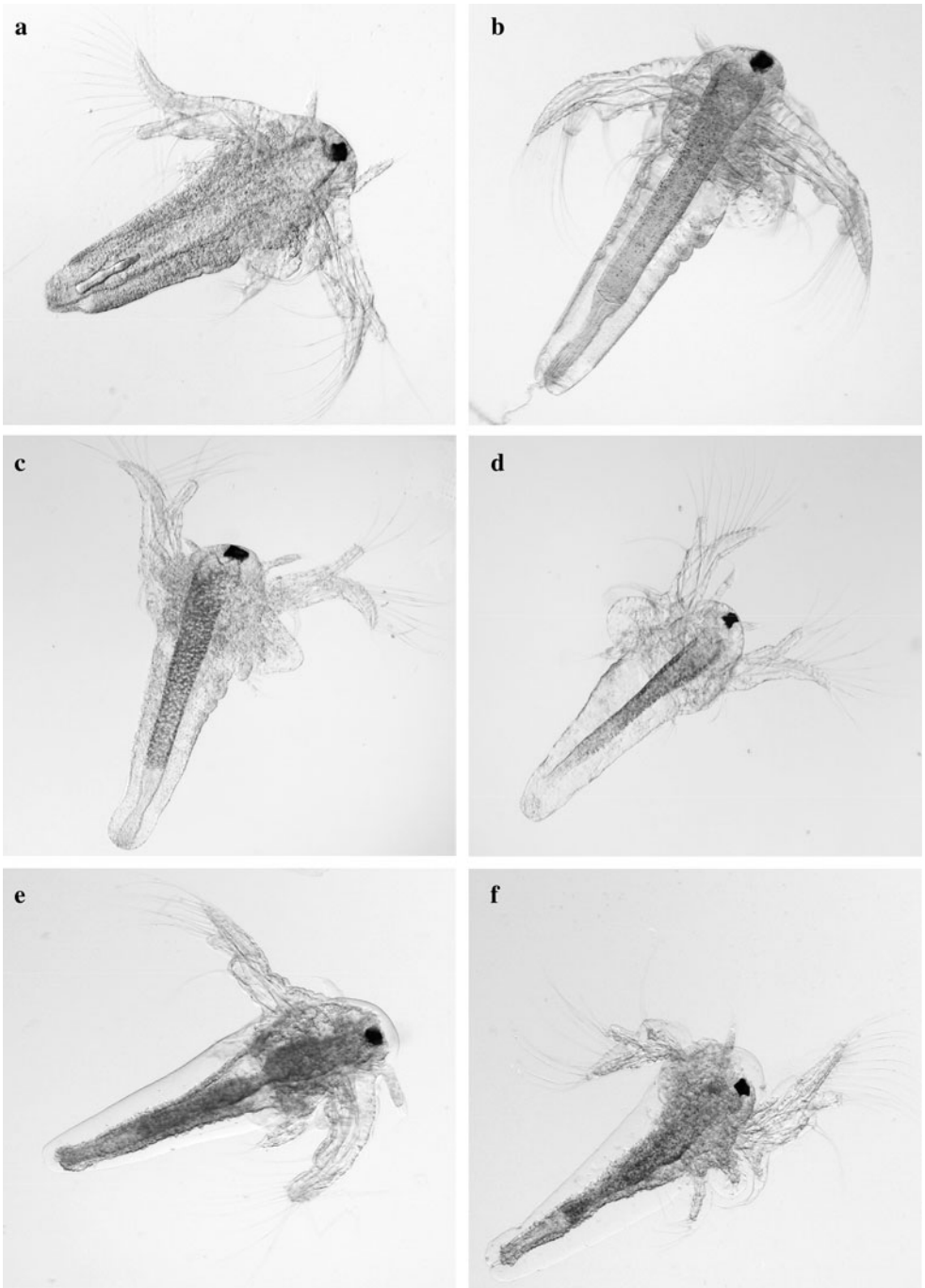

Fig. 1 **a** *A. franciscana* 0 h control group (100×). **b** *A. franciscana* 48 h control group (100×). **c** *A. franciscana* 48 h after exposed dose 250 Gy (100×). **d** *A. franciscana* 48 h after exposed dose 500 Gy (100×). **e** *A. franciscana* 48 h after exposed dose 1,000 Gy (100×). **f** *A. franciscana* 48 h after exposed dose 2,500 Gy (100×)

segmentation were also observed in our experiments. It is evident from Fig. 2 that the external dimension of the body width measured at all groups which were exposed to radiation doses was in practical terms identical to that of the control group. No statistically significant differences ($\alpha = 0.05$) were found among the above mentioned averages. On the other hand, variability increased. SEM was higher and it achieved to 9.5 compared to the control group in which SEM reached only 3.8.

While the body width did not change, the body length significantly changed depending on the radiation dose (Fig. 2). The body length in the control group achieved (900 ± 13.4) μm. In the group exposed to a dose of 0.25 kGy, the average body length was shorter (827 μm)

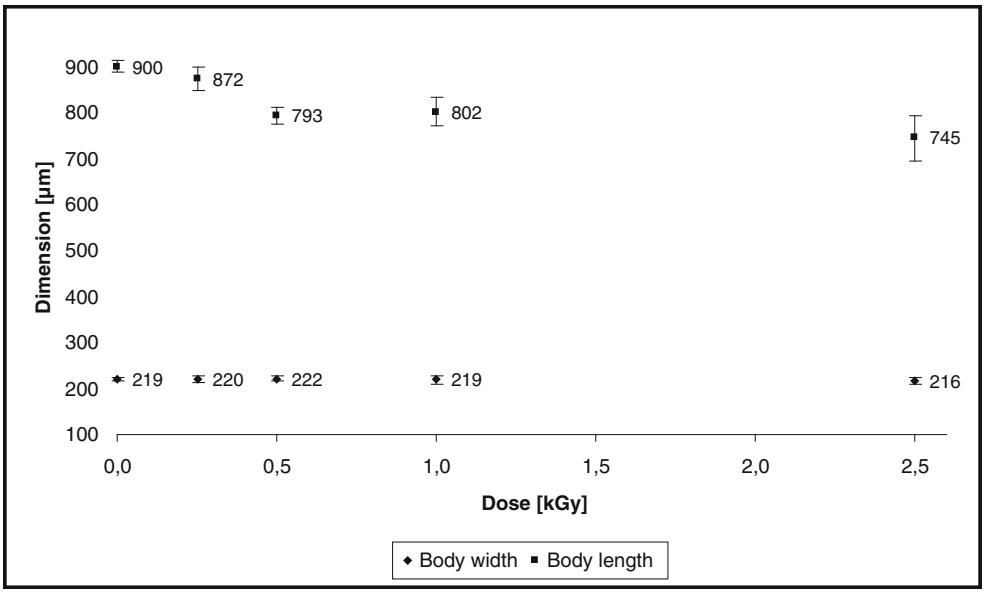

Fig. 2 Dependence of body width and body length (µm) on radiation doses received (kGy) in *A. franciscana* (mean ± SEM)

with remarkably higher SEM (25.4 µm). In the group exposed to a dose of 0.5 kGy, the body length achieved 793 ± 18.1 µm, and for a dose of 1.0 kGy the body length was 802 ± 30.2 µm. The shortest body length (745 µm) was achieved in the individuals who were exposed to a dose of 2.5 kGy. The highest SEM (49.1 µm) was observed in this group. The statistically significant differences ($\alpha = 0.05$) in body length depending on exposure were proved.

The ratio between the body length and the body width (Table 1) was statistically significant and dependent on the doses received ($\alpha = 0.01$). This ratio achieved a value of 4.13 in the control group, while 3.98 for a dose of 0.25 kGy and the ratio was reduced to the mutual ratio of 3.45 for a dose of 2.5 kGy.

A freshly hatched specimen of *A. franciscana* aged 40 h from the start of hydration (see Fig. 1a) was different compared to the following developmental stages.

In the developmental study of the second instar, segmentation especially in the thoracic part was typical. Phylopoda for breathing and food intake are formed additionally to the development of appendages. The morphological signs are showed in Fig. 1b at an individual from the control group of *A. franciscana* after 48 h from the start of the experiment (from the beginning of exposure). It is possible to observe significant segmentation in the thoracic part. It is also evident in the individuals exposed to a dose of 0.25 kGy (Fig. 1c) that segmentation in the thoracic part was much less developed, and moreover, it did not reach such a developmental level as with the individuals in the control group. The segmentation developed slower and was less noticeable in the individuals exposed to 0.5 kGy (see Fig. 1d). The segmentation in the individuals exposed to 1 kGy was nearly negligible and/or was fully missing (Fig. 1e). No segmentation was observed in the individuals exposed to 2.5 kGy (no development), see Fig. 1f. The majority of the individuals exposed to radiation doses were killed within 48 h after exposure.

A change of exoskeleton rigidity was also evident during preparation of the samples of exposed individuals. The exoskeleton was remarkably less rigid in the groups exposed to a dose of 0.5 kGy and more. Deformation of the animals occurred very frequently during preparation of the samples and removal of surplus liquid by filtration paper from below the cover glass. This was not caused by mechanical force (pressure), but only due to contact of the cover glass with the glass slide. However, this negative effect was not observed in the control group. In this case, the group exposed to a dose of 0.25 kGy was comparable with that of the control group.

The next observed morphological deviation was a change of intestine epithelium formation. Uninterrupted intestine epithelium in the control group was formed by a remarkable thick epithelial cell layer, see Fig. 1b. Remarkable flattening of intestine epithelium (see Fig. 1c) in the individuals exposed to a dose of 0.25 kGy was observed. Figure 1d shows *A. franciscana* exposed to a dose of 0.5 kGy. In this case, it was observed that the layer of intestine epithelial cells was remarkably thinner. Epithelial cells formed only a thin layer in the individuals exposed to a dose of 1 kGy, see Fig. 1e. The most remarkable differences among the individuals were evident and compared with Fig. 1f where *A. franciscana* was exposed to 2.5 kGy and aged 48 h after exposure. Due to the absence of development the complete internal structure was significantly different. Epithelial cells formed a very thin layer and in some places were missing.

Table 1 Dependence of the mutual ratios between epithelium width and intestine lumen, body width and intestine width, and body length and body width on radiation doses (kGy) in *A. franciscana* (mean ± SEM)

Dose (kGy)	Epithelium versus intestine lumen	Body width versus intestine width	Body length versus body width
0			
Mean	**0.85**	**2.96**	**4.13**
SEM	0.060	0.066	0.052
0.25			
Mean	**0.64**	**2.96**	**3.98**
SEM	0.059	0.070	0.067
0.5			
Mean	**0.52**	**2.95**	**3.60**
SEM	0.058	0.109	0.054
1.0			
Mean	**0.51**	**3.10**	**3.59**
SEM	0.039	0.149	0.058
2.5			
Mean	**0.45**	**3.39**	**3.45**
SEM	0.064	0.246	0.193
ANOVA			
F	**7.154**	1.467	**15.500**
F_{crit}	$F_{0.01} = 3.480$	$F_{0.05} = 2.447$	$F_{0.01} = 3.480$

F_{crit} critical value of ANOVA test for probability of 99 per cent ($F_{0.01}$) or 95 per cent ($F_{0.05}$)

With an increasing dose of ionizing radiation the ratio between the epithelial cell layer and intestine lumen was significantly reduced ($\alpha = 0.01$) (Table 1). In comparison with the control group (0.85) the ratio reduced from 0.64 in the group exposed to a dose of 0.25 kGy to the ratio of 0.45 in the group exposed to a dose of 2.5 kGy.

The results of ionizing radiation effect on intestine epithelium development in the *Artemia* crustacean genus were analogous to the damages of enterocytes in mammals. In general, it is well known that intestine epithelium cells are evident in vertebrates due to high mitotic activity in cells with very high radiosensitivity. The gastrointestinal syndrome was induced by the doses from 6 to 10 Gy in mammals, with average $LD_{50/30\ days}$ from 4 to 8 Gy. In the *Artemia* crustacean genus, $LD_{50/96\ h}$ was from 0.6 to 0.7 kGy (Dvořák and Beňová 2002). The complete disappearance of intestine epithelial cell layer occurred at doses higher than 1 kGy. However, the statistically significant differences in intestine epithelium width were already observed in our experiment from a dose of about 0.25 kGy.

The mutual ratio between the body width and the intestine width (Table 1) was variable from 2.95 to 3.39. The differences between their averages were not statistically significant. This ratio remained comparable for all doses with the control group.

The significant differences ($\alpha = 0.01$) for a reduction of intestine epithelium depending on the doses are evident in Fig. 3. Total intestine width did not manifest a statistically significant difference depending on doses. In the control group and for the majority of doses applied, this parameter was characterized by a comparable variability. The variability significantly increased for a dose of 2.5 kGy. Due to lowering intestine epithelium width, an increase of intestine lumen width for all doses applied was observed. The statistically significant increase of intestine lumen width was already detected for the lowest dose of 0.25 kGy.

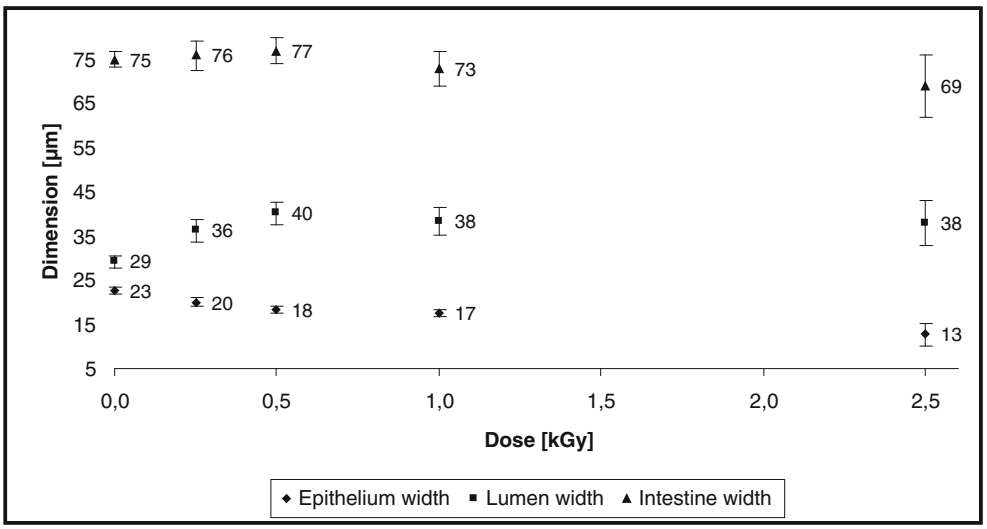

Fig. 3 Dependence of epithelium width, lumen width and intestine width (µm) on the radiation doses received in *A. franciscana* (mean ± SEM) (kGy)

Variability with increasing doses successively increased and the SEM reached the maximum for 2.5 kGy. A local disappearance of intestine epithelium in some places was observed for the highest doses of 1 and 2.5 kGy.

The morphological changes, the changes of dimensions and the mutual body ratios were observed in the individuals exposed to a dose of 0.25 kGy. The ratio between body length and body width and the ratio between intestine epithelium width and intestine lumen width were the most significant parameters which were used to observe the dependence on radiation doses. In general, it can be stated that we were able to observe a higher number of differences in crustaceans undergoing through developmental stages compared to the growth of vertebrate young. In this view, a biotest can partly substitute the conventional tests in vertebrates.

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