

Tolerance to gamma-irradiation in eggs of the tardigrade *Richtersius coronifer* depends on stage of development

Ingemar JÖNSSON,^{1,2*} Eliana A. BELTRÁN-PARDO,^{1,3} Siamak HAGHDOOST,¹ Andrzej WOJCIK,¹ Rosa María BERMÚDEZ-CRUZ,⁴ Jaime E. BERNAL VILLEGAS,³ Mats HARMS-RINGDAHL¹

¹Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, SE-106 91 Stockholm, Sweden;

²School of Education and Environment, Kristianstad University, SE-291 88 Kristianstad, Sweden; ³Pontificia Universidad Javeriana, Instituto de Genética Humana, Carrera 7 No 40-62, Bogotá, Colombia; ⁴Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional - CINVESTAV, Avenida Instituto Politécnico Nacional 2508, 07360 Mexico City, Mexico

*Corresponding author: ingemar.jonsson@hkr.se.

ABSTRACT

Tardigrades are known as one of the most radiation tolerant animals on Earth, and several studies on tolerance in adult tardigrades have been published. In contrast, very few studies on radiation tolerance of embryonic stages have been reported. Here we report a study on tolerance to gamma irradiation in eggs of the eutardigrade *Richtersius coronifer*. Irradiation of eggs collected directly from a natural substrate (moss) showed a clear dose-response, with a steep decline in hatchability at doses up to 0.4 kGy followed by a relatively constant hatchability around 25% up to 2 kGy, and a decline to ca. 5% at 4 kGy above which no eggs hatched. Analysis of the time required for eggs to hatch after irradiation (residual development time) showed that hatching of eggs after exposure to high doses of gamma radiation was associated with short residual development time. Since short residual development time means that the egg was irradiated at a late developmental stage, this suggests that eggs were more tolerant to radiation late in development. This was also confirmed in another experiment in which stage of development at irradiation was controlled. No eggs irradiated at the early developmental stage hatched, and only one egg at middle stage hatched, while eggs irradiated in the late stage hatched at a rate indistinguishable from controls. This suggests that the eggs are more sensitive to radiation in the early stages of development, or that tolerance to radiation is acquired only late in development, shortly before the eggs hatch, hypotheses that are not mutually exclusive. Our study emphasizes the importance of considering specific cell cycle phases and developmental stages in studies of tolerance to radiation in tardigrades, and the potential importance of embryonic studies in revealing the mechanisms behind the radiation tolerance of tardigrades and other cryptobiotic animals.

Key words: radiation tolerance, radio-resistance, tardigrades, *Richtersius coronifer*.

INTRODUCTION

Tardigrades are small invertebrate animals often inhabiting very exposed microhabitats such as mosses and lichens where they have adapted to undergo rapid and severe desiccation (Wright *et al.*, 1992). These populations tolerate a complete loss of water, even under high vacuum (Jönsson *et al.*, 2008), and may remain in a desiccated but viable state for several years (Guidetti and Jönsson, 2002; Rebecchi *et al.*, 2006; Jørgensen *et al.*, 2007). This ability is expressed at all developmental stages, from the egg to the adult tardigrade. Apart from showing an extreme tolerance to desiccation, tardigrades also belong to the most radiation tolerant animals. The extreme tolerance of tardigrades to ionizing radiation was first shown by May *et al.* (1964) using x-rays, and has been confirmed by recent studies where gamma-rays were used (Jönsson *et al.*, 2005; Horikawa *et al.*, 2006). Adult tardigrades survive several thousand Gray, with an LD₅₀ dose of 4-6 kGy observed 1-2 days after irradiation. They also tolerate high doses of UV radiation (Altiero *et al.*, 2011). Of particular interest is the fact that in all studies so far, including stud-

ies on X-ray, alpha, gamma and UV radiation, desiccated and hydrated tardigrades show similar survival. This shows that radiation tolerance in tardigrades is not restricted to mechanisms exclusive to the dry state. Recent studies have also shown that tardigrades are able to survive high linear energy transfer (LET) radiation, such as alpha particles (Horikawa *et al.*, 2006) and protons (Nilsson *et al.*, 2010) to a similar extent, or even better, than low LET radiation. This is striking because the relative biological effect (RBE) of high LET radiation is expected to be higher than for low LET radiation. Tardigrades have also been introduced as a model organism for studies under space conditions (Jönsson, 2007; Jönsson *et al.*, 2008). In the first exposure of an animal to the combined effect of space vacuum and cosmic/UV radiation in low Earth orbit (ca. 300 km asl) ever made, some tardigrades were able to survive and also reproduce (Jönsson *et al.*, 2008), confirming a remarkable ability to survive under extreme desiccation and radiation. However, the mechanisms behind the extreme radiotolerance of tardigrades remain unresolved.

Most previous studies on radiation tolerance in tardigrades have investigated adult animals, and although irradiated tardigrades moulted and laid eggs, the eggs seemed to be non-viable (Jönsson *et al.*, 2005; Horikawa *et al.*, 2006). However, Horikawa *et al.* (2012) recently reported a study on tolerance to alpha particles (^4H) in eggs of the tardigrade *Ramazzottius varieornatus* Bertolani and Kinchin, 1993, showing a dose-response in both hydrated and anhydrobiotic eggs. However, the dose-response was expressed at considerably higher doses in anhydrobiotic eggs compared to hydrated eggs.

We report a study on tolerance to gamma irradiation in eggs of the eutardigrade *Richtersius coronifer* (Richters, 1903). A clear dose-response was documented, but although some eggs were able to survive and hatch after doses up to 4 kGy, experiments in which developmental stage was controlled showed that radiation tolerance at high doses was restricted to eggs that were in late developmental stage.

METHODS

Study population

The eggs used in our study were from a parthenogenetic population of *R. coronifer*, inhabiting moss growing on carbonite rock fences at Öland's Alvar in south-east Sweden. This population has been the subject of numerous studies on environmental tolerance, including desiccation, freezing and radiation (Ramlov and Westh, 1992; Jönsson *et al.*, 2001, 2005).

Experiment with eggs collected from natural moss

For the first experiment, naturally laid eggs were extracted directly from the moss. The eggs were obtained by rehydrating dry moss in tap water and extracting the eggs by sieves of mesh size 40 μm and 250 μm . After rehydration the eggs were kept in mineral water at ca 5°C for 4-7 days before use in the experiment. Their developmental status could not be determined before the experiment and thus the eggs used in this experiment were most likely at various stages of development, from recently laid eggs to eggs close to hatching.

Egg samples were irradiated in 1.5 mL glass vials (one for each replicate) filled with distilled water and exposed to gamma irradiation (137Cs, Gammacell 1000; Isomedix, Inc., Kanata, Ontario, Canada) at a dose rate of 10 Gy/min and at room temperature. The following dose groups were used: 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1, 2, 3, 4, 6, 8, and 10 kGy. For each dose, three replicate samples were used, each with an average of 23 (18-38, SD=3.8) eggs. After irradiation the eggs were transferred to multi-well Petri dishes with 15 mm diameter cups filled with mineral water, and kept in a temperature controlled room at 15°C. Control samples were treated in the same way as irradi-

ated samples, apart from the irradiation procedure. All samples were then checked every 2nd day for hatchlings and new water was then added. Monitoring was continued for 75 days or until at least 20 days had passed since the last hatched egg.

Experiment with eggs laid under laboratory conditions - developmental stage controlled

Eggs for this experiment were obtained from adult *R. coronifer* kept under laboratory (but non-cultured) conditions. Newly laid eggs (180) were distributed equally into 12 Petri dishes (9 of which were used for the irradiated samples, and 3 for controls) with mineral water, 15 eggs in each dish, and kept in a refrigerator at 11°C until irradiated at specific developmental stages. Three developmental stages were used: early stage (2 days post-laying), middle stage (30 days post-laying) and late stage (50 days post-laying), with 3 replicates for each stage. The eggs were irradiated with a dose of 0.5 kGy, using the same gamma source as above, but with a dose rate of 6.74 Gy/min. After radiation, the eggs were transferred to 15 mm Petri dishes with mineral water. Monitoring and water changes were done every 2nd day, for the same time period as above. Control samples were treated in the same way as irradiated samples, apart from the irradiation procedure. The same set of controls were used for comparison with all three groups of irradiated samples, since the only aspect distinguishing the three treatment groups was the time during development at which they were irradiated.

Statistical analyses

Due to small sample sizes, non-parametric analyses with Kruskal-Wallis (KW) Analysis of Variance, Mann-Whitney U-test, and Spearman's rank correlation were used (SYSTAT 12; SYSTAT Software Inc., Chicago, IL, USA). Reported P-values represent two-tailed tests and $P \leq 0.05$ was used as criterion for statistically significant results.

RESULTS

Experiment with eggs collected from natural moss

As expected, the proportion of hatched eggs declined with dose, from ca. 70% in the controls to ca. 5% at the 4 kGy dose, resulting in an overall significant difference in hatching success among the dose groups (KW=37.0, $P < 0.001$, $df=13$; Fig. 1 and Tab. 1). No eggs hatched after exposure to doses above 4 kGy. However, the declining hatchability was not linear, but dropped rapidly over the lower dose range and then leveled off. Control eggs had significantly higher hatchability (ca. 70%) than all irradiated groups, and eggs irradiated at 0.1 kGy hatched significantly better than all groups above 0.3 kGy. Between 0.2 and 2 kGy, however, there was a marked plateau be-

($U=1.5$, $P=0.18$), while only one egg in the early developmental stage and no eggs in the middle stage hatched (Fig. 3). Thus, at the early and middle stages of development the eggs of this tardigrade species are very sensitive to radiation while in the late stage they are unaffected by gamma ray even at the relatively high dose of 0.5 kGy.

DISCUSSION

Common knowledge within radiation biology (*Law of Bergonié and Tribondeau*; Bergonié and Tribondeau, 1906), states that the radiosensitivity of a cell is directly proportional to its reproductive activity and therefore that

developing tissues will be most harmed by radiation. The results of our study are fully in line with this principle, showing a much higher sensitivity of tardigrade eggs than previously recorded for adult tardigrades. The irradiation of eggs from natural substrates showed a clear dose-response pattern in hatchability, with a steep decline in survival of eggs up to about 0.4 kGy followed by a constant hatchability around 25% up to 2 kGy, and then again a decline up to 4 kGy. The dose of 50% hatchability (LD_{50}) was considerably lower (48 Gy) than for previously investigated adults of the same species (4.7 kGy 18 h post-irradiation, 2.5 kGy 30 days post-irradiation; Jönsson *et al.*, 2005). Both the analysis of residual development time

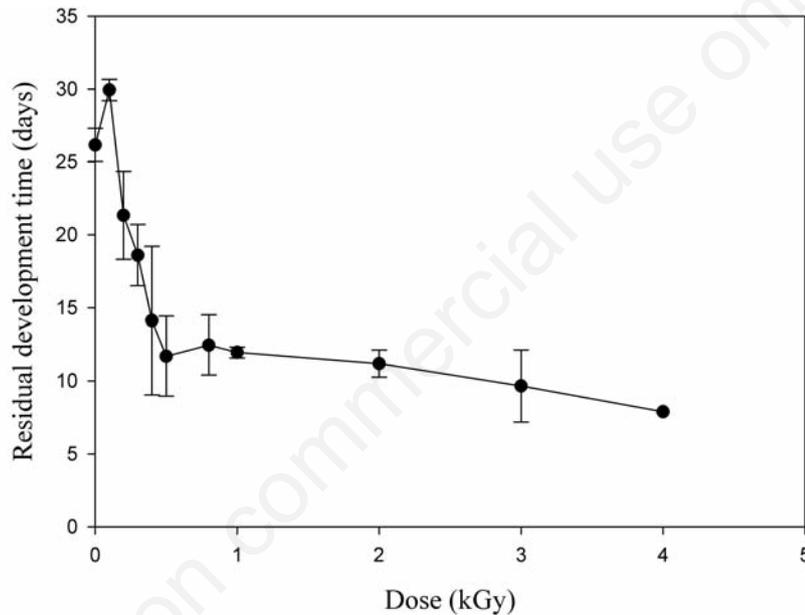


Fig. 2. Residual development time (in days) after gamma irradiation as a function of dose (in kGy) in eggs of the tardigrade *Richtersius coronifer*. Sample details as in Fig. 1.

Tab. 2. Mean hatchability (proportion hatched eggs) and residual development time from irradiation until hatch of *Richtersius coronifer* eggs exposed to gamma irradiation at doses between 0.1 and 4 kGy. Above 4 kGy no eggs hatched.

Dose (kGy)	Mean hatchability	SD	Residual development time (days)	SD
0	0.71	0.091	26.2	2.0
0.1	0.50	0.066	29.9	1.3
0.2	0.33	0.13	21.3	5.2
0.3	0.37	0.081	18.6	3.6
0.4	0.23	0.081	14.1	8.8
0.5	0.26	0.12	11.7	4.7
0.8	0.24	0.007	12.5	3.5
1	0.28	0.007	12.0	0.64
2	0.22	0.058	11.2	1.6
3	0.11	0.073	9.7	4.3
4	0.048	0.048	7.9	*

SD, standard deviation. *Residual development time based on one sample only.

after irradiation among eggs collected in natural substrates, and the experiment where development stage was controlled indicates that sensitivity of tardigrade eggs to gamma rays changes during development. Although the shorter residual development time of eggs hatching at high doses could result from a radiation-induced increase in rate of development, it seems more likely that only eggs that were in a late developmental stage hatched at high doses. This interpretation is also supported by the development stage experiment: while practically no eggs survived irradiation in the early and middle stages, in the late stage of development the eggs were unaffected by the 0.5 kGy dose. This may suggest that cell division and differentiation of the embryo was completed at the late stage, and that the organism therefore had achieved a state where it was much less affected by the radiation.

Takanami *et al.* (2003) showed that eggs of the nematode *Caenorhabditis elegans* (Maupas, 1900) had a higher hatchability when irradiated as meiotic pachytene cells (gametogenesis) compared to irradiation within early embryogenesis, and suggested that the resistance in pachytene stage was due to expression of enzymes like Rad51, involved in homologous recombination. Homologous recombination is only present in diploid cells, since it uses the sister chromatid as template for accurate exchange/repairing of the DNA damage (Moynahan and Jasin, 2010). The population of *R. coronifer* used for this

study is diploid ($2n=12$) and have automictic parthenogenesis with thelytoky (Rebecchi *et al.*, 2003). If the maturation of the oocyte is meiotic in *R. coronifer* and eggs are laid in metaphase I, as suggested by Rebecchi *et al.* (2003), the subsequent stages in meiosis and mitosis during early embryogenesis are expected to be more sensitive, because of the high rate of mitosis. Another possible explanation for the observed higher radiation tolerance in the late developmental stage could be that the efficient DNA repair system hypothesised for tardigrades (Jönsson *et al.*, 2005; Jönsson, 2007) is completed only towards the end of development. The high dose (0.5 kGy) survived by eggs in late developmental stage tend to support this explanation, possibly in combination with a lower mitotic activity. This dose is expected to generate a large amount of double strand breaks in the DNA (around 500 DSBs in rotifers; Gladishev and Meselson, 2008), which without an accurate and fast repair would lead to accumulated chromosome aberrations and mutations that would challenge genome integrity (Moynahan and Jasin, 2010). No studies so far have documented the molecular composition of tardigrade eggs in the course of development, but Schokraie *et al.* (2012) reported that about 24% of all proteins found in eggs of the eutardigrade *Milnesium tardigradum* Doyère, 1840 were unique to the egg stage and absent from the adult stage, but only eggs in the early developmental stage (24 h post-deposition, blastula stage)

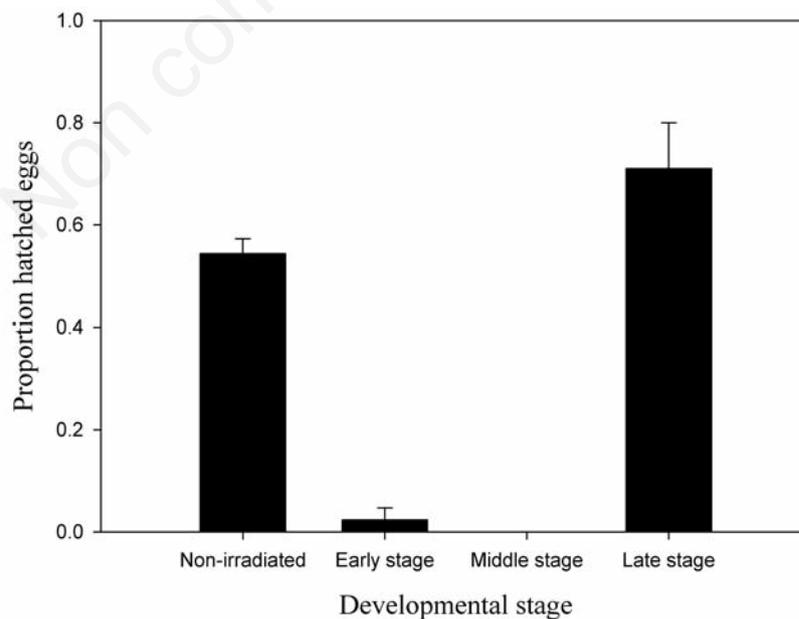


Fig. 3. Mean proportion (with standard error) of eggs that hatched after irradiation with 0.5 kGy at different stages of development, and for non-irradiated eggs, in *Richtersius coronifer*. Early stage=2 days post-laying; middle stage=30 days post-laying; late stage=50 days post-laying. For each developmental stage and controls, three replicate samples were used, each with 15 eggs. Estimated mean values (SD): control, 0.54 (0.051); early stage, 0.022 (0.038); middle stage, 0.0 (0); late stage, 0.71 (0.15).

were used. Also increased activity of antioxidant metabolism during development could be involved in the observed tolerance of tardigrade eggs. Such antioxidant activities have been reported in radiation and desiccation tolerant chironomid larvae after irradiation (Gusev *et al.*, 2010) and also in the eutardigrade *Paramacrobiotus richtersi* (Murray, 1911) in connection with desiccation (Rizzo *et al.*, 2010).

Only one previous study has presented data on the dose-response to radiation of tardigrade eggs. Horikawa *et al.* (2012) reported a study on alpha particle (^4H , high LET) irradiation of eggs of the eutardigrade *R. varieornatus*. The stage of development at irradiation was intermediate (2-3 days post-deposition, compared to the average total post-deposition development time of 5.7 days). Both anhydrobiotic and hydrated eggs were studied, and the latter showed a significantly lower tolerance than dry anhydrobiotic eggs. The dose-response pattern for hydrated eggs was similar to the one found in our study, with a steep decline in hatchability up to a dose of 0.75 kGy, and a much slower decline thereafter up to 2 kGy. However, the initial decline was not as steep as in the current study, and hatchability of control eggs was also higher (100%). Horikawa *et al.* (2012) reported an LD₅₀ value for hydrated eggs of 509 Gy based on a linear regression, considerably higher than our estimate of 48 Gy, but due to several differences in methodology and use of different kinds of radiation (alpha vs gamma) these estimates are not directly comparable.

A few other studies have evaluated tolerance of tardigrade eggs to radiation, without examining responses to dose. Jönsson *et al.* (2008) exposed eggs of *R. coronifer* and *M. tardigradum* to ultraviolet radiation+cosmic radiation in space, with no surviving eggs recorded. However, eggs that were sheltered from UV-radiation and exposed only to space vacuum and cosmic radiation hatched normally. In the same space flight, Persson *et al.* (2011) reported no hatching of *R. coronifer* eggs exposed to cosmic radiation at 4 Gy while eggs of *M. tardigradum* exposed to the same condition showed 100% hatching. In neither of these exposures in space was the stage of development of exposed eggs controlled.

The results of our study on radiation tolerance are in line with previous experiments on desiccation tolerance in tardigrade eggs. Schill and Fritz (2008) exposed eggs of the tardigrade *M. tardigradum* at five different stages of development to desiccation at different rates, and recorded the hatch rate. Eggs that were desiccated at the earliest stage (24 h post-deposition) had lowest hatch rate, while eggs desiccated late in development (stages 4 and 5) had the highest rate. Eggs at mid-developmental stages had intermediate hatch rates. Although our results suggest that radiation tolerance is achieved only in the late stage of development, the general pattern is clear: radiation toler-

ance show similar patterns as desiccation tolerance. This supports the view that tolerance to desiccation and radiation rely on the same mechanism, and that the latter may be a by-product of selection for the former (Jönsson, 2003).

CONCLUSIONS

Our study is one of the first on radiation tolerance in tardigrade eggs, and the first evaluation of tolerance in different stages of egg development. The study shows that eggs of the tardigrade *R. coronifer* achieve high tolerance to gamma irradiation only at the late stage of development. This result may arise either from a decline in mitotic activity over the period of development, development of biochemical mechanisms related to protection or repair of DNA, or a combination of both. Further studies on radiation tolerance and molecular patterns during egg development of tardigrades may contribute to resolving the mystery of how tardigrades manage to survive conditions that are lethal for most other eukaryotes.

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