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In vitro Study of the Survival, Reproduction and Morphology of *Daphnia pulicaria* irradiated with a Low Energy Laser

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ABSTRACT

Daphnia is a genus of crustaceans that is representative of freshwater communities. The species exhibit a high sensitivity to a wide range of toxic compounds so that they have been used internationally as biomonitors in toxicity tests to evaluate ecosystem conditions such as water quality. It is also a model genus in genetics, epigenetics and reproductive ecology. In this work, we used *Daphnia pulicaria* as a model to measure the effects of low-energy laser irradiation on survival, reproduction, and morphology variables of parental organisms and their offspring. We used (1) a single clone line of organisms to eliminate interindividual genetic variability; (2) individuals from more than 50 generations after the clone line was established, and offspring from the third brood onwards to dissipate maternal and epigenetic effects, and (3) neonates, those individuals of the species that have less than 48 hours of life, because they are the most sensitive stage to optical stimuli. We analyzed number of deaths, longevity, age at first reproduction, number of offspring per week, number of total offspring during all their life cycle, body size, size of the antennules, and length of the apical spine of the 4th and 5th brood of the irradiated individuals, who were exposed to a blue laser stimulus of 405 nm for 25 minutes with a power of 40 mW at a distance of 50 cm, compared to those of the control (non-irradiated) group.

1. Introduction

Daphnia is worldwide considered a model organism in genetics, epigenetics and reproductive ecology experiments aside from being an excellent toxicity bioindicator in aquatic environments since they are the main representatives of the freshwater communities. By exhibiting high sensitivity to a wide range of toxic compounds, they have been internationally used in toxicity tests to measure and evaluate the conditions of an ecosystem. Besides, its adaptability and culture maintenance in the laboratory are relatively simple allowing the performance of quick and economic tests [1]. The reproductive cycle of Daphnia is one of its principal and greatest advantages as it is directly related to environmental stimuli. If the environmental conditions are ideal, Daphnia will carry out its reproduction through a parthenogenetic or asexual cycle, whereas when the environmental conditions are disturbed, its reproductive cycle will change to a sexual one.

Daphnia magna, *Daphnia exilis*, *Daphnia pulex*, *Ceriodaphnia dubia* and *Moina macrocopa* are used as toxicological models to measure the median lethal concentration (LC_{50}), particularly of agricultural and industrial chemical products [2]. The toxins tend to accumulate in the flea body, and their subsequent effect is reflected as oxidative stress or damage. This property can be quantified through specific biomarkers, including antioxidant enzymatic defenses such as superoxide dismutase and catalase activity which are considered the first and second line of defense against reactive oxygen species. Oxidative damage can also be determined through the level of lipid peroxidation since lipids -especially unsaturated ones- are the molecules most susceptible to oxy-radicals. Moreover, in aquatic species, there is a close relationship between the increase in lipid peroxidation and oxidative deterioration [3].

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Previous studies have exposed Daphnia to various toxins and evaluated their response to oxidative stressors as those generated from cyanobacteria blooming, artificial mixtures of cyanotoxins [3], exposition to wastewater effluents under ozonation treatments, and to advanced oxidation processes for removing drugs and their metabolites [4,5]. The results showed that exposure to toxic compounds might cause genetic or epigenetic changes in future generations [6,7]; such changes evaluated through differences in survival rate, longevity, growth, reproduction, and morphology. Another study conducted by Kushibiki *et al.* [8] showed that low-energy laser therapy induces a signaling cascade of second messengers which leads to the production of reactive oxygen species in irradiated cells, especially with a 405 nm blue laser. The increase in reactive oxygen species, adenosine triphosphate, and/or adenosine monophosphate concentrations seems to be to the fact that the components of the respiratory chain can absorb the energy of the laser-induced photon in the mitochondria. This phenomenon also promotes cell proliferation and cytoprotection. The benefits experimented by the cell and the production of reactive oxygen species depends on the exposure time, pulse frequency and dose delivered.

2. Method

2.1 Generation and Maintenance of Daphnia Pulicaria Clone Line

Crustaceans of *Daphnia pulicaria* Forbes, 1893 species (Crustacea, Cladocera) were collected from a freshwater body in the area of Alto Lerma, State of Mexico, Mexico. Specimen acclimation to laboratory conditions was carried out according to Official Mexican Standard NMX AA-087-SCFI-2010 [9] and the protocol described by Martínez-Jerónimo *et al.* [2]. A clonal line was generated from a single mother by parthenogenetic reproduction and maintained in these standard conditions for more than 50 generations. Andrewata and Burggren [10] demonstrated that after three generations, there is a dissipation ("washout") of epigenetically induced phenotype changes caused by exposure to an environmental stressor of the parental generation, thereby we infer that our specimens were suitable for the experiment. The clone line was maintained in an area free of toxic substances, immersed in reconstituted hard water (according to the referred Mexican Standard) at a temperature of 20 ± 2° C, dissolved oxygen of 2 mg/L, hardness ($CaCO_3$) of 250 ± 25 mg/L, and pH of 8.0 ± 0.2. Water was replaced three times a week. Specimens were exposed to a light intensity from 600 lx to 1000 lx and a photoperiod of 16L : 8D. They were fed with suspended cells of the microalgae *Desmodesmus sp.* three times a week at a concentration of 750 000 to 800 000 cells/mL.

2.2 Experimental Protocol: Laser Irradiation Exposure

For evaluating the possible effects of blue laser irradiation on the daphnias, two experimental groups were formed. The first was non-irradiated (control group), and the second (treatment group) was exposed acutely to the irradiation of a solid-state blue laser (wavelength of 405 nm) for 25 minutes with a power of 40 mW at a distance of 50 cm (Figure 1). The luminous intensity generated in this treatment was 10 *mW/Cm*^{2} Both groups consisted of ten *Daphnia pulicaria* neonate sisters from the same mother between 24 to 48 hours old. For washing out any possible epigenetic effect, sisters from the third brood of the same mother were used [11].

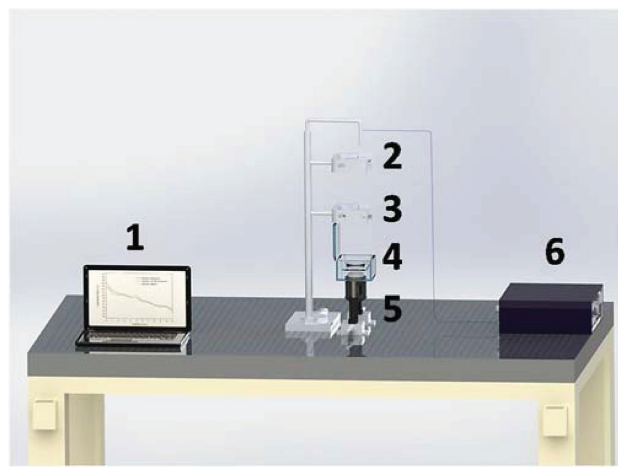

Figure 1. Experimental set. Daphnia samples were placed inside a temperature-controlled irradiation chamber (1 and 4), through an optical arrangement (2 and 3). They were irradiated with a 405 nm blue laser (6). Images were captured using a camera adapted to a microscope (5).

2.3 Experimental monitoring

The 10 neonates of the control and irradiated groups were separated and identified individually in beakers containing one daphnia per 10 ml of reconstituted hard water. The water was previously standardized with the necessary food concentration; and pH, temperature, electrical conductivity, and total dissolved solids were measured with a multiparametric meter (HANNA HI98129) as described in the NMX-AA-087-SCFI-2010. A database was generated for each individual of the control and treatment groups, registering the number of deaths, longevity, age at first

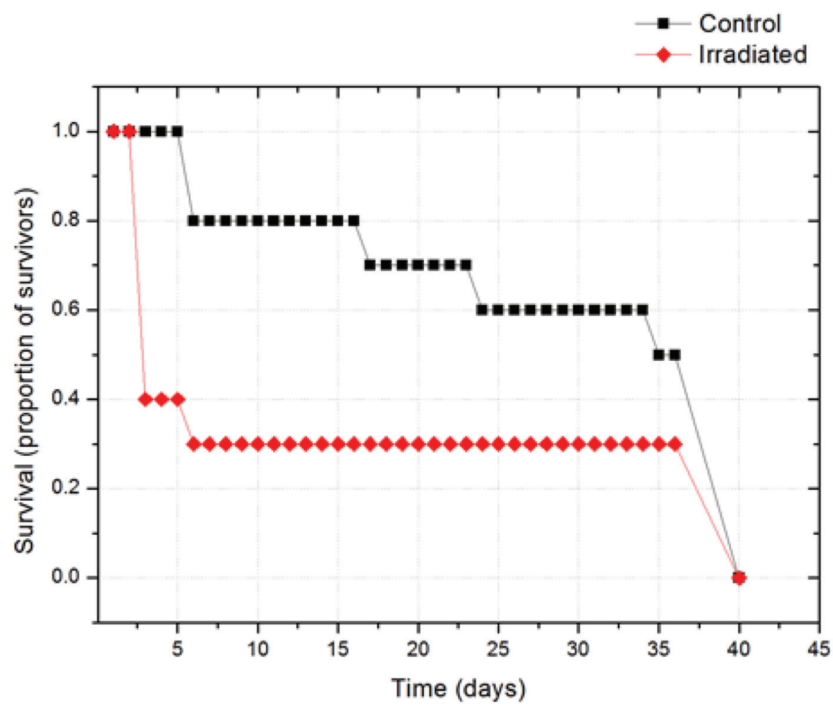

Figure 2. Control and irradiated survivorship curves.

reproduction, number of offspring per week, and number of total offspring during all their life cycle. Morphology variables including body size, size of the antennules, and length of the apical spine were also registered for each individual and their offspring.

2.4 *Statistical Analysis*

A graphical comparison with the age (days) vs. survivorship (number of survivors) as dependent and independent variables respectively was elaborated. The resulting curves were contrasted with the type I, II, and III population ecology models described by Stiling [12]. The effect of laser irradiation on longevity, reproduction, and morphology variables was analyzed by means and median comparisons tests. When the data analyzed had a normal distribution and the sample size was big enough, a student "t" was applied (t). Otherwise, a nonparametric two-sample comparison, Mann-Whitney (W), was carried out.

3. Results and Discussion

3.1 *Survival Rate*

The effect of blue laser irradiation on survival is very noticeable; it can be observed in the proportion of survivors as a function of time (Figure 2). The lethal dose was identified for 70% of the individuals. Under normal conditions, the control group exhibited a constant survival rate similar to a type II curve [12]; which shows that individuals die naturally at a constant rate at all ages. In this group, just 80% of the individuals reached sexual maturity. The treatment group exhibited a type III survival curve [12]; where there is high mortality in the first three days after irradiation, and the survivors reached adulthood and achieved reproduction.

3.2 *Longevity*

The total number of days lived was 41 to 43 in the control group, in the experimental group only 30% of the total sample reached 42 days without variation. In this variable, there were no significant differences ($W = 7.5$, $df = 5.3$ and $p = 0.84$).

3.3 *Reproduction*

In the individuals that reached sexual maturity, the age at first reproduction did not present differences between the individuals of the control group and those irradiated; all had their first brood at 15 days after being born. The total number of offspring was affected by radiation in a negative and significant way ($t = 3.1$, $df = 6$ and $p = 0.01$), the control group had 119-145 offspring, and the irradiated group had 102-115.

3.4 Morphology

No apparent morphological changes were detected in the experimental individuals, however, in the offspring of the irradiated individuals (F1) the morphological changes were very significant in neonates born in the 4th and 5th broods and consistent with those of different mothers. In them, the group where the mothers were irradiated had a significant effect when compared with the control group; this factor produced a decrease in body size ($t = 2.1$, $df = 29$ and $p = 0.02$). Likewise, there was a tendency to reduce the size of the antennules, although this was not statistically significant ($t = 1.6$, $df = 29$ and $p = 0.1$). On the other hand, the irradiation produced a very significant increase in the length of the apical spine ($t = 2.84$, $df = 29$ and $p = 0.004$) as seen in the photographs of figures 3 and 4. The conservation of morphological changes, in the 4th brood, suggest a possible genetic change as proposed by Andrewartha and Burggren [10] and Burggren [6,11], since the epigenetic effects vanish after the third brood and if such effects persist, the probability that they are genetic is very high.

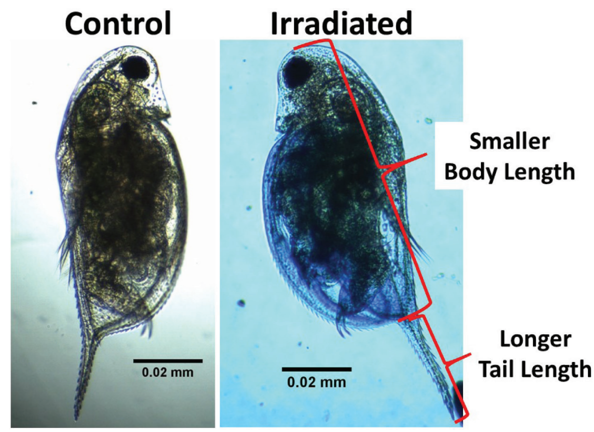

Figure 3. 4X optical microscope images of control and exposed neonates of *Daphnia pulicaria* showing the morphological measurements changes induced by blue laser irradiation.

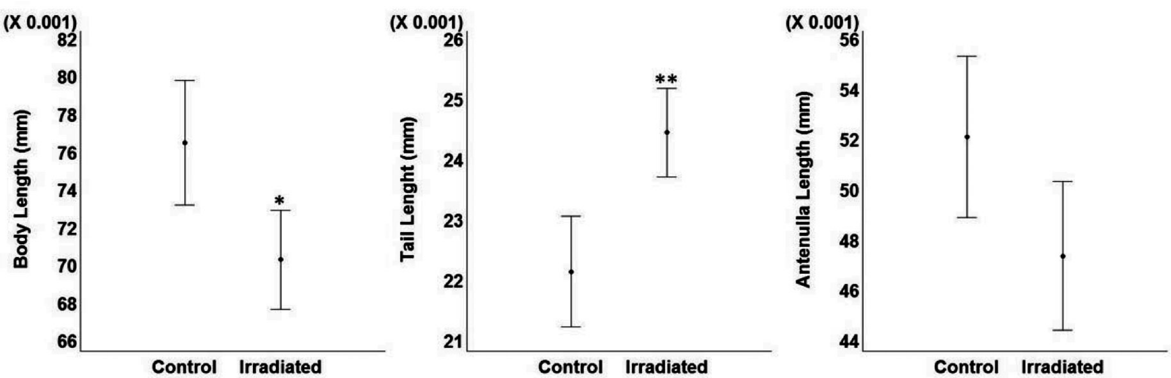

Figure 4. Morphological comparisons of F1 neonates obtained from *Daphnia pulicaria* control and irradiated mothers. The lines show 95% confidence intervals and dots represent the group average. Asterisks denote statistically significant differences between groups, * means $p<0.05$, and ** means $p<0.01$.

4. Conclusions

The irradiation with a solid-state blue laser (wavelength of 405 nm) for a time of 25 minutes and with a power of 40 mW produces significant changes in the survival, reproduction, and morphology of *Daphnia pulicaria.* This dose was lethal for 70% of the irradiated neonates and decreased the total number of offspring of the surviving individuals. The morphology was affected in neonates of the 4th and 5th brood of different irradiated mothers, where there was a decrease in the size of the body, a significant increase in the apical spine and a tendency to reduce the size of the antennule.

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