Estimation of the DNA damage in *Girardia tigrina* (Girard, 1850) after exposure to radiation under extreme conditions

Estimativa dos danos do DNA em *Girardia tigrina* (Girard, 1850) após exposição à radiação em condições extremas

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ABSTRACT

The use of planarians as experimental models has been increased worldwide especially due to their high capacity of tissue regeneration and ease of laboratory maintenance. The current study aims to assess the genetic damage of *Girardia tigrina* after exposure of stressors in conditions such as: critical temperature, critical pH, hypergravity (4.4G) and low power laser (685nm). 18 specimens of *G. tigrina* were used, the extreme conditions mentioned were experimental group with 3 specimens each, and 3 for the control group, all specimens were submitted to the Comet Assay after exposure of stressors, and control group with no extreme condition exposure. The extreme stimuli of critical temperature and pH, hypergravity and low power laser irradiation, caused genetic damages to the *G. tigrina*, for hypergravity and low-power laser stressors the damage was higher in the pharyngeal and post-pharyngeal regions of the animal with a greatest area of comet tail dragging for the post-pharyngeal region. The extreme stimuli of critical temperature and pH, hypergravity and low power laser irradiation caused genetic damages in planarians

G. tigrina, especially in samples from the pharyngeal and post-pharyngeal regions of the flatworms body assessed by the Comet Assay.

Keywords: Planaria, pH, Temperature, Hypergravity, Low-power laser

RESUMO

As planárias têm sido cada vez mais utilizadas como modelos experimentais devido à sua elevada capacidade de regeneração e facilidade de manutenção em laboratório. O estudo atual visa avaliar os danos no material genético de *Girardia tigrina* através da exposição a uma série de condições de estressoras tais como: temperatura crítica, pH crítico, hipergravidade (4,4G) e laser de baixa potência (685nm). Foram utilizados 18 indivíduos de *G. tigrina*, e para cada condição extrema acima mencionada, foram utilizados 3 amostras para o grupo experimental e 3 para o grupo controle, sendo essas submetidas ao teste cometa. Os estímulos extremos de temperatura crítica e pH, hipergravidade e irradiação laser de baixa potência, causaram danos ao material genético da *G. tigrina*. Nos testes com hipergravidade e laser de baixa potência, ocorreram danos no material genético do grupo experimental, nas regiões faríngeal e pós-faríngeal, com uma maior área de arrastamento da cauda do teste cometa na região pós-faríngeal. Os estímulos extremos de temperatura crítica e pH, hipergravidade e irradiação laser de baixa potência causaram danos no material genético de *G. tigrina*. As planárias submetidas à hipergravidade e ao laser de baixa potência mostraram danos no material genético, nas regiões faríngeal e pós-faríngeal, mostrando um extenso arrastamento da cauda do teste cometa para a região pós-faríngeal.

Palavras-chave: Planaria, pH, Temperatura, Hipergravidade, Laser de baixa potência

1 BACKGROUND

Planarians have been increasingly used as an experimental model because, in addition to their regenerative capacity, they respond quickly to experimental manipulation and are easy to maintain in the laboratory [1, 2]. Studies with this model organism have attempted to fill in gaps in the understanding of the behaviour of stem cells under extreme conditions, contributing to the understanding of both of the organization and, more generally, to the reaction of tissues in such conditions.

Planarians are an animal group distributed worldwide and which occupy a variety of thermally distinct freshwater habitats. Different types of planaria can inhabit a variety of habitats, including marine and terrestrial environments [3, 4]. Water temperature is an important factor involved in the distribution and abundance of different species of triclades platyhelminths [5].

The locomotor activity of animals subjected to thermal stress can be analyzed by the method known as “Critical Thermal Maximum” (CTM), developed by Cowles and Bogert [6]. Different species can be evaluated because the behavioral response involves

a sequence of progressive slowness or immobility and irregular movements or contortions. The use of extreme thermal conditions has allowed researchers to assess the tolerance and physiological parameters of a wide variety of organisms [6, 7, 8]. In ectothermic organisms other than planarians, species the fish Bathygobius soporator and Parablennius marmoreus, shrimps Palaemon northropi and Hippolyte obliquimanus, and crabs Eurypanopeus abbreviatus and Menippe nodifrons [9], all show similar findings with symptoms such as loss of balance and spasms. Corroborating the actions described by Díaz and Bückle [10], for the fish Ictalurus punctatus, and by Oliveira et al. [11] for freshwater planarians *Girardia tigrina* and *Girardia sp*., the animals showed spasms and loss of balance in response to extreme thermal conditions. Girardia sp. is unable to survive in waters below pH 3.0 [11], a fact also recorded made for *Girardia dorotocephala, Cura foremanii, Dendrocelopsis vaginatus* and asexual specimens of *G. tigrina* planaria by Rivera and Perich [12].

Before death, limnic planarians exposed to extreme temperature conditions show contortions, slowness, peeling of the epidermis and loss of the ability to move properly [5, 11, 12, 13].

The hydrogen ionic point (pH) of lentic ecosystems influences the reproductive cycle and the environmental adaptation capacity of many free-living invertebrates.

Animals such as freshwater planarians are able to withstand wide pH variations by homeostatic regulation of their internal metabolism. However, limit points exist, perhaps determined by alterations to physiology and behavior [12, 11].

Various animal groups show the capacity for regeneration, though at different intensities. For example, while humans easily regenerate a cut epidermis, hydras are able to regenerate an entire anterior region after amputation, while salamanders and some insects can regenerate lost limbs [15].

Planarians have a high capacity for regeneration, possessing totipotent stem cells called neoblasts throughout their existence. They are capable of regenerating after severe amputations to the body structure, doing so differentiating any bodily tissue from stem cells located in the mesenchyme. This capacity makes them the focus modern research to understand the mechanisms involved and their possible application in the study of human stem cells. Regeneration in planaria involves the generation of new tissue at the wound site through the proliferation of cells, resulting in the formation of the blastema, and remodelling of pre-existing tissues, in order to restore symmetry and morphology [16].

The exposure of simple organisms with well-marked regenerative capacity to hypergravity can help develop understanding of what happens to human and animal astronauts in different gravitational environments. Studies by Campos-Velho [17] show that specimens of *G. tigrina*, retained their regenerative capacity in hypergravity environments, with the posterior fragments showing a significantly higher average growth than the control group. In the case of human beings, even a brief exposure to hypergravity causes displacement of body fluids to the lower body, reducing blood pressure in the head and thorax causing and may cause damage to the nervous system, affecting both memory and visual perception [18].

The use of low power lasers to treat wounds in human tissue and their interaction with the wound site increases cell metabolism and vascularization [19, 20], while the effects of low power laser stimulation depend on the wavelength, dose, and power of the light used in the irradiation process [21].

The Comet Assay (CA) or Single Cell Gel Electrophoresis, is a fast and efficient technique to quantify lesions, damages and detect the effects of DNA repair, with several advantages over biochemical and cytogenetic tests, including the capacity to use a small number of cells including those that are not dividing [22]. As a result, it is widely used in studies of toxicological genetics and environmental monitoring [23], including studies with limnic planarian populations, e.g. evaluation of toxic and genotoxic effects of copper sulphate [24, 25]. The quantification of data made visible in a Comet Assay is made by microscopic analysis, by counting 100 nucleoids per repetition, which in turn can be defined as the genetic material supported by the nucleus proteins, whose membrane has been lysed [26].

The aim of the current study was to use the comet assay evaluate DNA damage in *G. tigrina* individuals submitted to extreme conditions of pH, temperature, hypergravity and low power laser.

2 METHODS

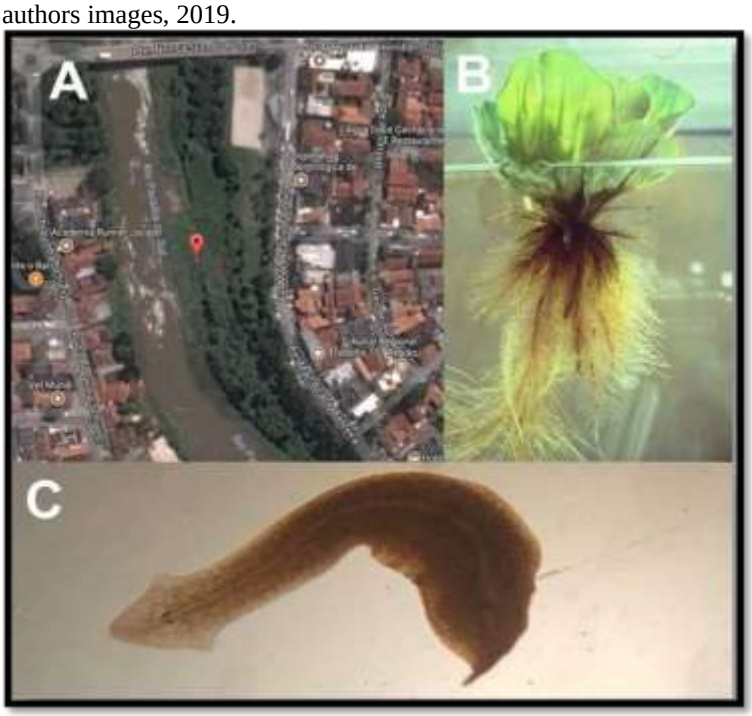
2.1 EXPERIMENTAL APPROACHES

A total of 18 *G. tigrina* specimens were used, derived from clonal lines maintained by the Planaria Research Laboratory (LAPLA) in University of Paraiba Valley (UNIVAP).

The founding batches of *G. tigrina* came from collections made in 2015 and 2016 at coordinates 23º18'18.0"S 45º58'40.3"W, on a stretch of the Paraíba do Sul River (in the

city of Jacareí, Sao Paulo State, Brazil), in roots of Pistia stratiotes, an aquatic plant (Figure 1). Clonal strains were acclimatized in the laboratory in ways that maintained their anatomical characteristics.

Figure 1: A- View of the stretch of the Rio Paraíba do Sul (Jacareí city, Sao Paulo State, Brazil) in which the colony founding stock was collected; B- example of Pistia stratiotes; C- *G. tigrina*). Source: Google Earth-Mapas and authors images, 2019.



2.2 CULTIVATION, SELECTION AND AMPUTATION

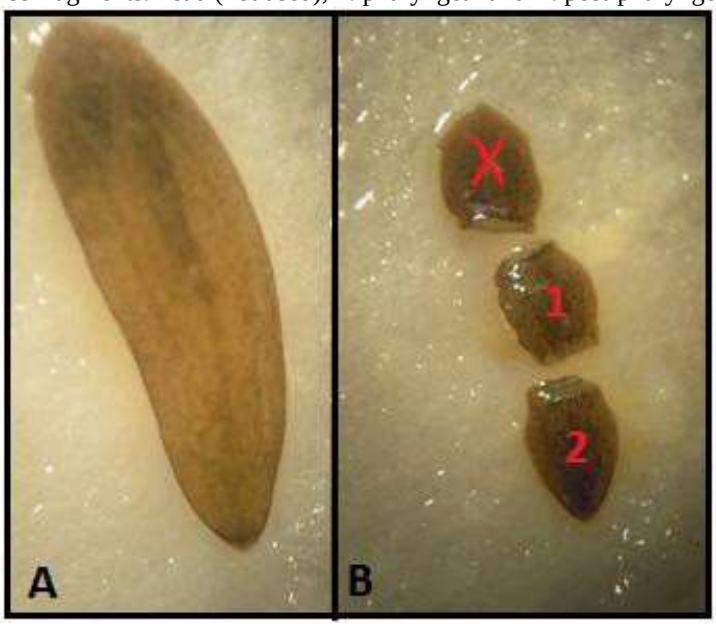
Specimens selected for the study were intact, with complete and undamaged morphologies, and body sizes that ranged from 0.5 to 1 cm. The planarians were placed in plastic containers (5 cm in diameter x 7 cm in height) containing 10 mL of maintenance water, free of chlorine or chemical agents (pH atsource 6.3 ± 0.1), and collected in the city of Jacareí, São Paulo, Brazil
(23°12'38.2"S, 45°57'56.6"W).

Following a protocol designed to ensure that was no interference from food remains in the animal's digestive tract, participating specimens were feeding suppressed for a period of 15 days before the beginning of the experiment [27, 28]. Individuals were housed individually in bottles under natural light.

The specimens submitted to hypergravity and low power laser underwent an amputation process, in which they were positioned with the ventral surface facing upwards then placed on ice under filter paper to anaesthetise them. They were sectioned in two regions: pharyngeal or median and post-pharyngeal or posterior (Figure 2).

According to Adell et al. [29], planarians amputated at any level along the anteroposterior axis can regenerate the head, but the regeneration rate decreases as one approaches the anterior region. Consequently, the head fragment was not used as its low rates of regeneration could potentially have interfered with the results.

Figure 2: Amputation of *G. tigrina*. A - whole specimen with the ventral region facing upwards 40 x, B - example cut into three fragments: head (not used), 1: pharyngeal and B: post-pharyngeal, 10 x.



Specimens submitted to the conditions of critical pH and critical temperature were not amputated due since apoptosis events related to injury recovery associated with such experimental sectioning could interact with extreme conditions tests, further damaging animal tissues [30, 31].

2.3 EXTREME CONDITIONS

2.3.1 Critical Thermic Conditions

The species *G. tigrina* is known to have a Maximum Thermal Critical Point (TCP) of 33ºC, with metabolic changes occurring after 27ºC, such as slow movements, mucus release, changes in metabolic fluids, peeling of the epidermis and, finally, death [11]. In the current study three specimens of *G. tigrina* were exposed for 3 hours at an extreme temperature (33ºC), incubated in a refrigerated greenhouse, in the absence of light, for acclimatization according to the protocol of Rivera and Perich [12].

2.3.2 Crítical Hydrogeniation Potencial (pH) condition

For the experimental critical pH group, three *G. tigrina* specimens were selected, placed in containers containing 30 mL of maintenance water with adjusted pH to 4.0, with 2 M NaOH or 1 M HCl, following the protocol in Oliveira et al. [11], a study in which planarians exposed to pH 4.0 were found to have suffered greater injury than those exposed to an extreme alkaline pH. For the control group, three specimens remained in maintenance water (pH at source 6.3 ± 0.1) at 20ºC, for 18 hours. To measure the pH of the water, microprocessed digital pH meters were used.

2.3.3 Hypergravity and Irradiation with Low-power Laser

For each of these experiments, 6 fragments were selected, three for the control group and three for the experimental group. The fragments were placed in 50 ml tubes and four fragments were distributed per tube, with maintenance water and

centrifuged. To reach 3.3 G, a speed of 550 rpm (revolutions per minute) was used.

The tubes were run for nine consecutive days, centrifuging for 9 hours/day, with 15 hours/rest/day, at an average temperature between 18ºC and 24ºC ± 1ºC, ideal for maintaining the fragments, and the same temperature condition as the control group experienced. When removed from hypergravity, the fragments were regenerated.

After centrifugation and an additional 24-hour interval, the specimens were distributed in pots with containing maintenance water and, to reduce the region of mobility of the animals these had an area of 2 cm², thus ensuring a greatest coverage by the laser. Specimens were irradiated in the dark with Low Power Laser, 685nm, dosage of 1.25/cm², power 13.3 ± 0.3 mW/cm² for 3 minutes.

2.4 COMET ASSAY

For the comet assay, 18 specimens were selected and distributed three experimental blocks, with three individuals in each of the two options:

A) Critical Thermal Condition (33ºC) and Control Group (ambient C);

B) Condition of critical hydrogen potential (4.0 pH), and Control Group;

C) 3.3G hypergravity (9 days/9h/day) 24-hour rest followed by Low Power Laser radiation (685nm) for 3min, and Control Group.

The Comet Assay was carried out, using the adaptations of Guecheva et al. [24] and Prá et al. [32]. After being subjected to extreme conditions indicated in A, B and C, the specimens were packed in eppendorfs, isolated and mechanically and chemically disintegrated using a Pasteur pipette, performing pison-like movements several times in 0.48% trypsin (W/V), and pH 7.3 phosphate-saline buffer (PSB) for 5 minutes. After centrifuging for 5 minutes at 1,000 rpm, the supernatant was removed and the remaining cells resuspended in 500 µL of PBS. Then, 20 microliters of suspension was added to 60 µL of low melting point agarose, in 0.5% PBS, and immediately deposited on the surface of a slide pre-coated with normal agarose (0.75% in PBS), and coverslips added. After remaining for 10 minutes at 4ºC for solidification, the coverslips were carefully removed with the aid of a hypodermic needle and transferred to a lysis solution. Negative and positive internal controls were then performed as a standardization procedure [33], for the positive, 100 µL of hydrogen peroxide (0.1%) was added for 20 minutes. Lysis buffer treatment occurred for 1 hour at 4ºC, composed of 2.5 M NaCl, 100mM Na2EDTA, 0.4 M Tris HCL (pH 10), and Triton X-100 and 1% dimethyl sulphoxide-DMSO and were added immediately before use. Prior to electrophoresis, slides were immersed in alkaline buffer prepared with 300mM NaOH and 1mM EDTA (pH 13.0) for 20 minutes at 4 ºC. Electrophoresis was performed at 300 mA (25 V) for 20 minutes, using the existing buffer. Slides were neutralized with 0.4M Tris (pH 7.5) with minutes five of incubation, interspersed with 3 washes in distilled water. Then they were stained with ethidium bromide (2 µL/ml), covered with a coverslip and photographed individually with an epifluorescence microscope (Leica DMLI). The images obtained were analysed using OpenComet version 1.3 [34].

3 RESULTS

3.1 COMET ASSAY

Analysis of the comet assay carried out on planarians subjected to thermal stress, pH extremes, hypergravity and low power laser indicated damage to the genetic material of the cells was present compared to the control group. Results were evaluated using an automated tool for image analysis of comet tests (Open Comet program version 1.3) [34] so as to reduce possible errors can occur when analysis is performed manually. Damage to *G. tigrina* DNA for each stressing stimulus is illustrated in the extent of tail generated by the comet assay (Figures 3 to 6).

Figure 3: Analyses by Comet Assay of cells from *G. tigrina* subjected to pH experimental tests. The Control shows no comet tail drag, indicating little or no alteration in the genetic material of the cell. At the critical pH (4.0) point, comet's tail drag (in red) is present suggesting severe damage to the cell's genetic material compared to other analyses.

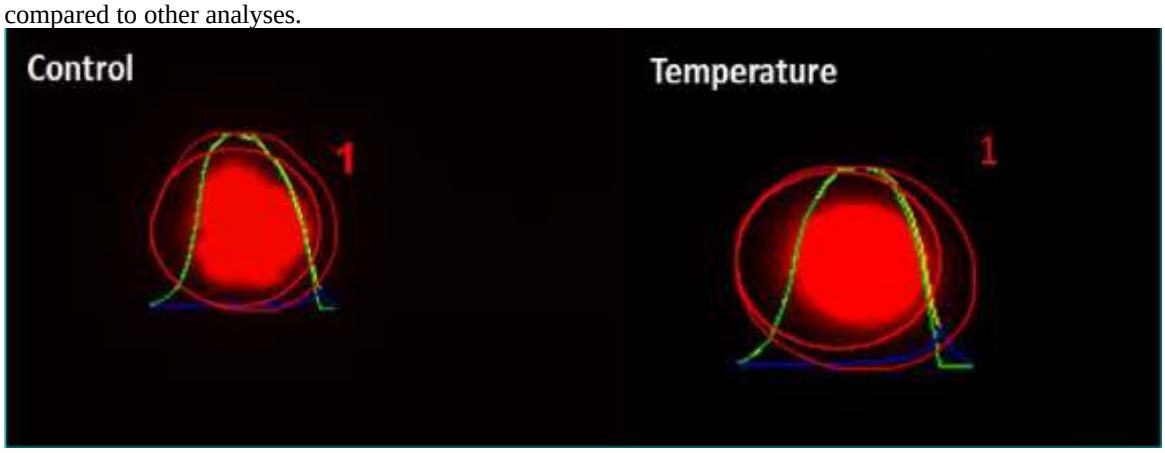


Figure 4: Analyses by Comet Assay of cells from *G. tigrina* subjected to pH experimental tests. The Control shows no comet tail drag, indicating little or no alteration in the genetic material of the cell. At a critical temperature (33ºC), a small drag in the genetic material can be seen in the experimental sample cells, indicative of little damage.

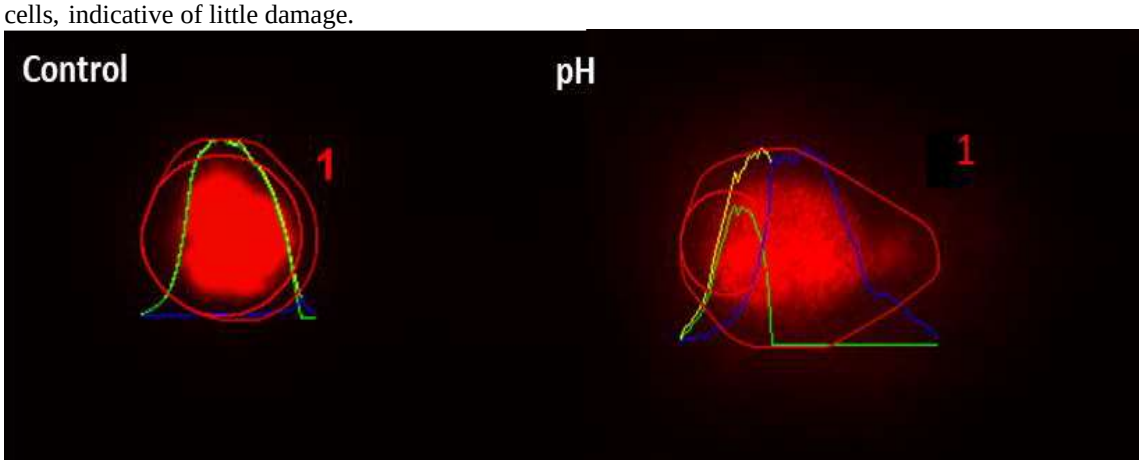


Figure 5: *G. tigrina* pharyngeal fragment for the laser control group shows no tail drag, indicating no change in genetic material. However, a pharyngeal fragment of *G. tigrina* for laser experimental group, the tail shows red drag indicating damage to the genetic material of the analysed cell.

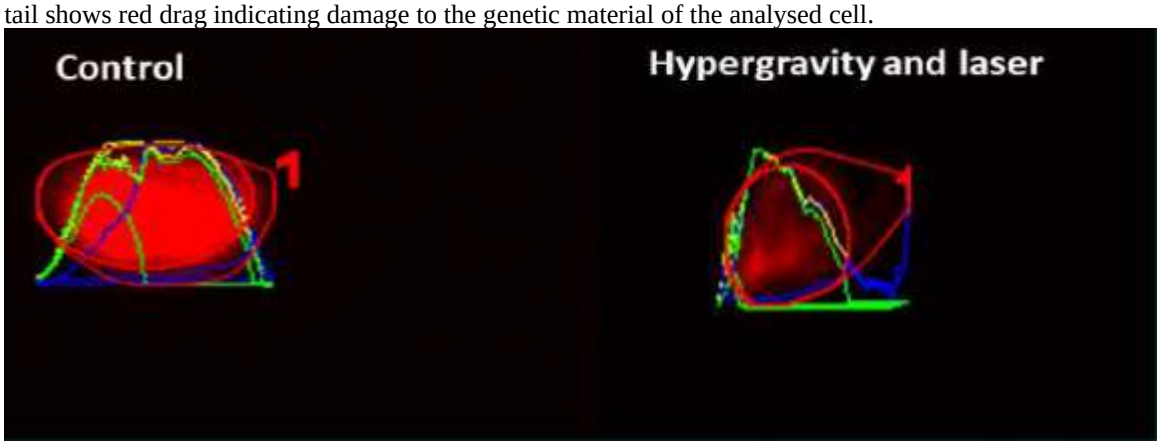
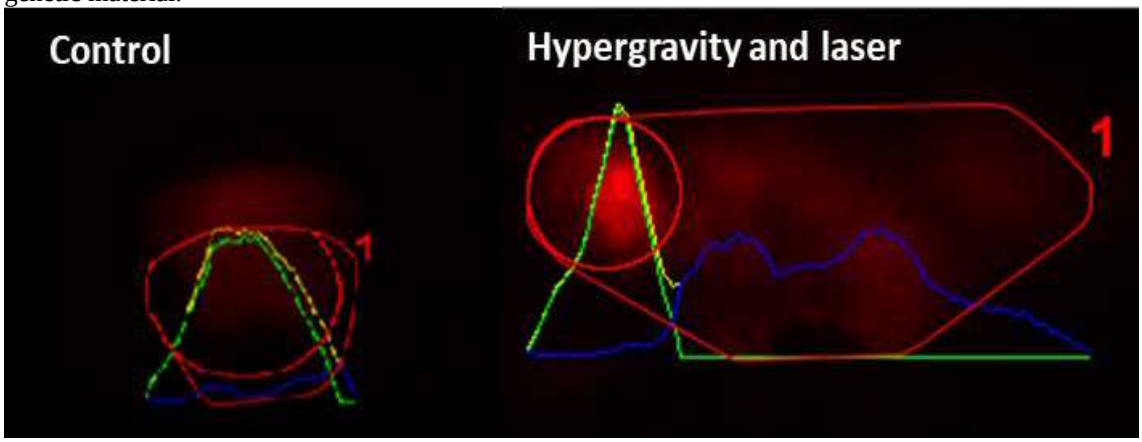


Figure 6: Post-pharyngeal fragment of *G. tigrina* for the laser control group, showing the area of comet tail drag for this group is small, so indicating no significant damage to the genetic material. In the *G. tigrina* post-pharyngeal fragment from the experimental group, tail dragging is observed, indicating damage to the genetic material.



Graphical analysis shows a difference in the percentage of DNA observed in the tail area of each fragment, this may have occurred because slides were prepared from a pool of cells, and one region may contain a greater number of cells in relation to another. In the indicates graph showing the changes in genetic material, presence in the upper area, as opposed to the median, greater damage in the DNA of the cells analysed. While presence of in the lower part in the median, indicates a lower percentage of changes in the genetic material. In the current study specimens exposed to the critical pH had a lower percentage of genetic material damage than for critical temperature, while both control groups (critical pH and critical temperature) had a lower percentage of damage to genetic material when compared to the experimental groups (Figure 7). In the group exposed to hypergravity and low power laser, there was a higher percentage of DNA damage in the post-pharyngeal fragment compared to the pharyngeal. For both fragments, the experimental group showed a higher percentage of damage than the control groups (Figure 8).

Figure 7: Comet tails area (%), caused by DNA dragging, thus indicating the degree of change for the experimental group: critical pH, critical temperature and control.

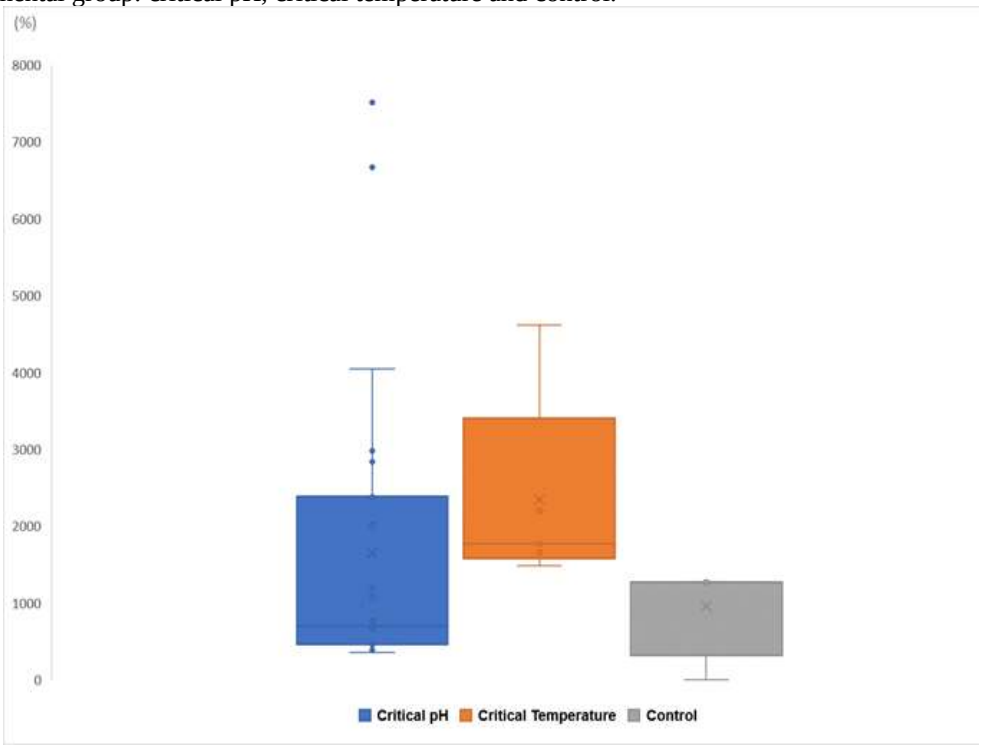
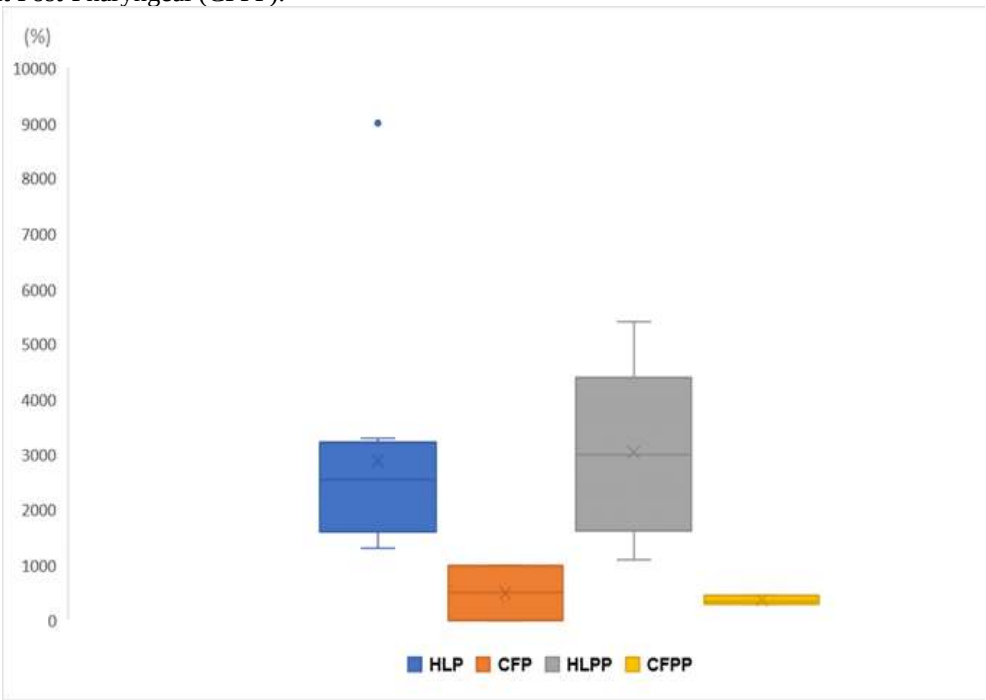


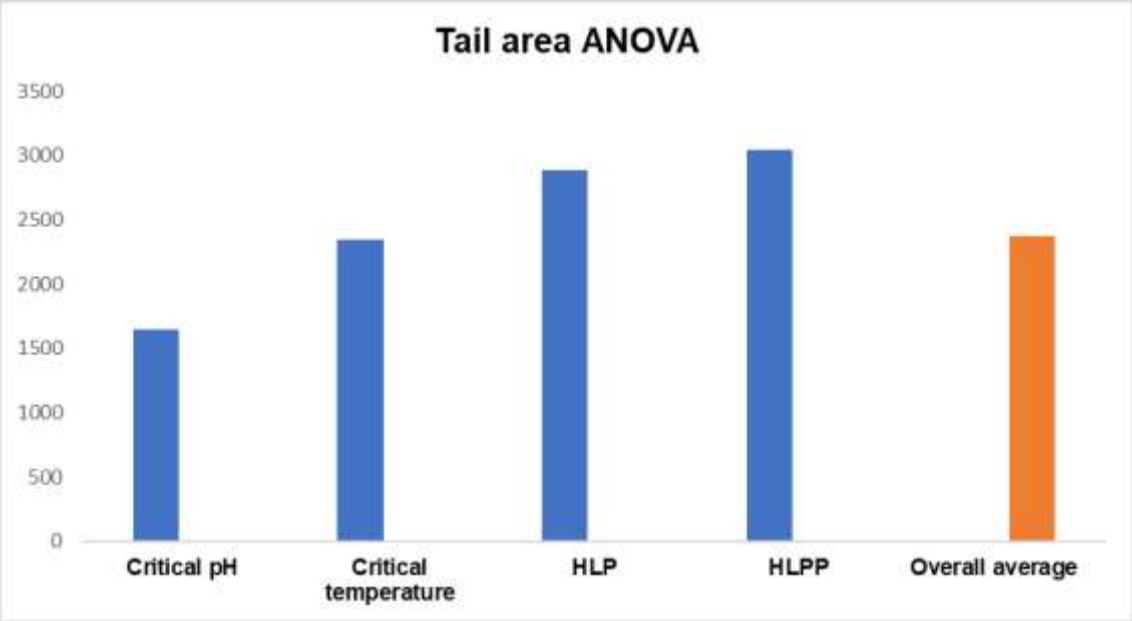
Figure 8: Comet tail areas (%), caused by DNA drag and indicating the level of alteration by the experimental group in comparison to the control group in each fragment: Hypergravity Laser Pharyngeal (HLP), Control Fragment Pharyngeal (CFP), Hypergravity Laser Post-Pharyngeal (HLPP), Control Fragment Post-Pharyngeal (CFPP).



3.2 STATISTICAL ANALYSIS

The data obtained were subjected to statistical treatment using SISVAR software version 5.6 [35, 36]. For analysis and interpretation of results, ANOVA (Analysis of Variance) was used with the TUKEY test, $p < 0.05$ (Figure 9).

Figure 9: Analysis of Variance of the area of comet tails (%), caused by the dragging of DNA for all experimental groups: critical pH, critical temperature, Hypergravity and laser pharyngeal fragment (HLP) and Hypergravity and Laser Post-Pharyngeal fragment (HLPP), the overall average of observed damage with the interaction between the treatment averages was recorded. The Tukey test was applied at the 5% probability level ($p < 0.05$, Tukey).



4 DISCUSSION

Ghecheva et al. [24] used in their work the comet assay in Girardia schubarti freshwater planarians and CF1 strain mice to analyse and compare the toxicity and genotoxicity of Copper Sulphate in these animals and alterations in the DNA of both species. Such a study both shows the effectiveness of this test in the analysis of genetic changes of planarian and mouse DNA, as well as affirming the sensitivity of these animals to Copper Sulphate. The comet assay in planarians is described in the literature as a genotoxicity evaluation method and, so far, there are no published studies that used the comet assay for planarians to assess DNA after submission to extreme conditions, such as those used in the present study. In addition to the specific utility of the results obtained, this makes the current study pioneering for this evaluation form of regeneration in the limnic planarian *G. tigrina.*

Examination of the Comet Assay images (Figures 3 and 6) show that they resemble those described by Lee and Steinert [37], who noted that the comet assay had

several applications, including observing DNA lesions, and apoptosis, and reaffirmed that a variety of chemical compounds, physical agents and other stimuli such as radiation for example, can damage the DNA of living cells. Such lesions at the level of genetic material, if not repaired, can lead to a cascade of biological events that may eventually result in the reduction of the population of the species.

The objective of the present study, was to observe the existence and dragging of the comet's tail and to measure DNA damage through the parameter “Tail area”. The observed formation of a comet tail indicated lesions in the genetic material of planarian cells exposed to extreme stimuli, such as critical temperature (Figure 3) and critical pH (Figure 4). Genotoxicity studies, mainly with copper sulphate [24, 25], ecogenotoxicology and biomonitoring [32, 38], used other parameters such as tail length, tail power and tail moment to assess damage.

Platyhelminths exposed to extreme pH conditions showed a smaller area of tail formation, which indicates less damage to the genetic content of the cells compared to the other experimental groups (Figure 9) – a conclusion supported by the fact that the obtained damage value was below the general average obtained via analysis of variance of comet tail area ($F = 2.542$, $p < 0.05$). The findings for extreme pH and temperature conditions corroborate the observations of Oliveira et al. [11], who analyzed scanning electron microscopies of *G. tigrina* previously stressed with extreme temperatures and pH, and concluded that the damage to the epidermal structure was greater for the critical thermal condition than for extreme pH. The planarian *G. tigrina* is distributed worldwide, and inhabits limnic ecosystems with a great variety of different pHs, between 4.0 and 9.0 [12, 39]. This great capacity for pH adaptation in different aquatic environments may be responsible for the lesser damage observed in comet tests during the current study.

Planarians exposed to a critical temperature of 33ºC showed greater DNA damage than those exposed to a critical pH of 4.0 (Figure 7), as indicated by comets with more extensive tail areas. Following induction of stressful thermal conditions in an aggregate of Pavona divaricata coral cells by incubating colonies in greenhouses at 31ºC for 10 hours and a control group at 25ºC, Nessa and Hidaka [40] conducted comet tests, which corroborated the current study, as they found greater damage to the DNA of coral cells exposed to a critical temperature of 31ºC.

Studies by Searby et al. [41] showed that gravity influences the mechanical environment inside the tissues, and can alter the organism's homeostasis, but not lead to death. After exposing 30 *G. tigrina* individuals sectioned them in the post- auricular

region to hypergravity (3.3G) for nine days, Pinto [42] demonstrated that there was the fragments showed subsequent regeneration, without occurrence of death. When sectioned in the head, pharyngeal and post pharyngeal and then subjected to hypergravity (3G) for a 13-day period, planarian Schmidtea mediterranea completely regenerated from fragments and appeared indistinguishable from respective controls. A similar event also occurred in the present study, since the fragments of the experimental group exposed to hypergravity of 3.3 G for nine days also regenerated [29].

De Sousa et al. [43] submitted amputated specimens of Schmidtea mediterranea to hypergravity (8G) and microgravity (speed of 10°/s), and observed that changes in gravity produce several genetic changes in planarians, although these changes have no apparent consequences, for example during regeneration, and may be due to transcriptional changes, cellular malfunction and excessive proliferation. The same study also reports that, after six days of exposure to altered gravitational conditions, only a few genes appear unregulated, indicating that they can adapt to long-term exposure to altered gravity. which is likely to be one of the reasons why planarians regenerate correctly, since the initial few days of the regenerative process are crucial for the correct formation of new structures. In the present study, it was found that individuals submitted to hypergravity regenerated without apparent damage, but with damage to their genetic material. This occurred for both for the pharyngeal and post-pharyngeal regenerating fragments.

Ermakova et al. [44] used different wavelengths (463, 520, 635, 420, 533 and 638.5nm) of low power LED laser in their work and reports that the use of planarians as a biological model allowed a detailed study of their mechanisms, including molecular level, proliferation and differentiation of stem cells in vivo.

Following irradiation of *G. tigrina* with a low power laser (660nm), performing three treatments composed of: animals individually irradiated with 14 sessions of 1 minute in duration (treatment 1), with 14 sessions of three minutes in duration (treatment 2) and non-irradiated (control), Lopes et al. [45] concluded that laser radiation with the applied power density was not lethal for *G. tigrina*.

According to Gyori et al. [34], cellular DNA is constantly attacked by chemical agents, nvironmental factors, ultraviolet rays and radiation, with corresponding damage being associated with the etiology of various diseases, such as cancer. The comet assay for DNA analysis and possible damage DNA induced by chemical or physical agents is extremely important, being performed by calculating the tail area generated in this assay,

making it possible to indicate changes and damage to the DNA of the cells analyzed. The combinations of the hypergravity and laser conditions were shown in the results of its comet tail, as extreme factors for the genetic material of *G. tigrina*.

This study opens new questions and directions for future technological and scientific approaches on the physiological adaptability of freshwater planarians to extreme conditions such as pH, temperature, hypergravity and low power laser radiation.

5 CONCLUSION

The extreme stimuli of critical temperature and pH, hypergravity and low power laser irradiation caused damage to the genetic material of the planarian *G. tigrina*. The flatworms exposed to extreme pH conditions showed the formation of a smaller comet tail area and, consequently, indicated that less damage to the genetic content of cells had occurred compared to experimental groups in the study. Animals exposed to critical temperature showed greater DNA damage compared to those exposed to critical pH, but less damage than those exposed to critical hypergravity conditions and low-power laser irradiation. In this context, it is notable that planarians have a great capacity to adapt to pH in different aquatic environments, which may explain why this test produced the lowest level of damage when as analysed by the comet assay. Planarians submitted to hypergravity and low-power laser showed damage to the genetic material, with samples from the pharyngeal and post- pharyngeal regions, showing extensive tail dragging from the comet to the post- pharyngeal region.

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