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Environmental Enrichment Effects on Zebrafish *(Danio rerio)* Fecundity, Fertility and Survival Rate

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Declaro que a presente dissertação é da minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras de atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

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Abstract

Environmental enrichment is a method of mimicking the animals' natural habitat to improve welfare, behaviour and ultimately reproduction. In theory, it can have positive influences on reproductive outcomes for all species, including fish and specifically zebrafish. Zebrafish (*Danio rerio*) is a widely and globally used species for laboratory investigation and, particularly regarding reproduction, shares many similarities with mammals making it a good research model. The benefits of environmental enrichment for this species when used in experiments remain unclear. This study aimed to discover effects of environmental enrichment in zebrafish fecundity and fertility rates and egg survival until hatching. To that purpose, zebrafish adult breeders were exposed to environmental enrichment in the form of gravel and plastic plants for periods of 7 and 14 days. The results obtained showed that this form of environmental enrichment had no significant effect on zebrafish fecundity and fertility rate, and egg survival. One exception was found for survival at 72h pf, which was found to be significantly higher for eggs produced by groups exposed to environmental enrichment. It was also concluded that different times of exposure, 7 and 14 days, had no significant effect on the fecundity and fertility rate and in egg survival. However, these results are only valid for the tested type and layout of environmental enrichment, making it necessary to test other materials and combinations in the future to allow robust conclusions.

Keywords: *Danio rerio*; egg survival; environmental enrichment; fecundity; fertility; husbandry; reproduction; zebrafish.

Resumo

O enriquecimento ambiental é um método de imitar o habitat natural dos animais a fim de melhorar o bem-estar, o comportamento e fundamentalmente a reprodução. Em teoria, pode ter influências positivas nos resultados reprodutivos de todas as espécies, incluindo peixes e, especificamente, o peixe-zebra. O peixe-zebra (*Danio rerio*) é uma espécie amplamente e globalmente usada para investigação laboratorial e, principalmente no que diz respeito à reprodução, compartilha muitas semelhanças com os mamíferos, tornando-o um bom modelo de investigação. Os benefícios do enriquecimento ambiental para esta espécie quando usada em investigação continuam pouco claros. Este estudo teve como objetivo descobrir os efeitos do enriquecimento ambiental nas taxas de fecundidade e fertilidade do peixe-zebra, bem como na sobrevivência dos ovos até à eclosão. Para tal, reprodutores adultos de peixe-zebra foram expostos a enriquecimento ambiental na forma de gravilha e plantas plásticas por períodos de 7 e 14 dias. Os resultados obtidos mostraram que esta forma de enriquecimento ambiental não teve nenhum efeito significativo nas taxas de fecundidade e fertilidade do peixe-zebra e na sobrevivência dos ovos. Uma exceção foi encontrada para a sobrevivência às 72h pf, que foi significativamente maior para ovos produzidos por grupos expostos ao enriquecimento ambiental. Concluiu-se também que os diferentes tempos de exposição, de 7 e 14 dias, não tiveram efeito significativo nas taxas de fecundidade e fertilidade, e na sobrevivência dos ovos. No entanto, estes resultados são válidos apenas para o tipo e *layout* de enriquecimento ambiental testados, tornando necessário testar outros materiais e combinações no futuro para alcançar conclusões robustas.

Palavras-chave: *Danio rerio*; enriquecimento ambiental; fecundidade; fertilidade; peixe-zebra; reprodução; sobrevivência de ovos.

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1. Introduction

1.1. Zebrafish

1.1.2 Taxonomy and Morphological Characteristics

Zebrafish, *Danio rerio*, is part of the freshwater fish family *Cyprinidae,* which is the vertebrate family with the highest species richness because it contains about 44 danionin species (Kalueff & Cachat, 2011). Its geographical distribution includes South and Southeast Asia, but the highest diversity of species occurs in north-eastern India, Bangladesh and Myanmar (Kalueff & Cachat, 2011).

Figure 1: Zebrafish male. Source: (Zfin.org)

Figure 2: Zebrafish female. Source: (zebrafishlab.be)

Danio rerio, first described by Francis Hamilton in 1822, is a small fusiform fish (Figure 1 and 2), usually under 120 mm, with a peculiar pattern of alternating light and dark horizontal stripes (Spencer et al, 2008). Its mouth is oblique and turned upwards, while the lower jaw stands out further than the upper one (Kalueff & Cachat, 2011). Also, zebrafish eyes are central and cannot be seen from above (Spencer et al, 2008).

The identifying characteristics of the species are two pairs of barbels, between five and seven longitudinal dark blue stripes, from behind the operculum extending to the caudal fin, and an uncompleted lateral line spread to the pelvic fin base (Kalueff & Cachat, 2011). While the dorsal fin is dark blue and white at the upper edge, the anal fin is striped like the rest of the body (Spencer et al, 2008).

Similarly to other teleosts, zebrafish melanophores can be moved as a response to different stimulus, which can be used for camouflage and for signalling, as the fish usually darkens when showing aggressive behaviour (Kalueff & Cachat, 2011).

The colouration does not vary much between sexes, but males (Figure 1) often have larger anal fins with a stronger yellow colour (Kalueff & Cachat, 2011). To make an infallible distinction between juvenile sexes, it is necessary to dissect the fish since the most reliable identification feature is a small genital papilla in front of the anal fin (Kalueff & Cachat, 2011). However, a more practical distinction can be made by observing body shape since females (Figure 2) tend to have wider and rounder bodies (Conradsen et al, 2015; Kalueff & Cachat, 2011).

1.1.2 Habitat

As far as environmental conditions, zebrafish tolerate a range of temperatures from below 10ºC up to 40ºC, a pH from 6 to little below 10, conductivity from 10 to 271 mS/cm, multiple substrates and vegetation, and elevations from sea level to 1500 m of altitude (Aleström et al, 2019).

However, zebrafish prefer to live in shallow, slow-moving, or bodies of still water with vegetation, silty substrate and much sunlight, frequently close to rice fields; these are a source of fertilizers that increase zooplankton, which is a large part of the zebrafish diet (Kalueff & Cachat, 2011).

Zebrafish are found in a variety of environments that may include small streams, rice paddies and rivers which contain different types of vegetation such as aquatic plants or overhanging vegetation but also substrates that may include gravel, mud and sand (Stevens, 2021).

1.1.3 Sex differentiation and gonad development

Zebrafish gonads all begin to develop as ovaries. Still, in males, around 5 to 7 weeks post-hatching, they start to differentiate and around the third month they became normal testes, with the timeline varying between different strains or environmental conditions (Kalueff & Cachat, 2011).

The process of sex determination is currently known to be genetically induced. However, most studies about that focus on laboratory breed fish, which have lost the natural sex determination locus that wild strains have, making it difficult to make a general conclusion (Kossack & Draper, 2019).

Despite that, sex ratio in nature seems to be 1:1, while in laboratory strains it is irregular and probably influenced by other factors (Kossack & Draper, 2019). There is some evidence that diet or growth rate may affect this process, with females usually being the ones that develop faster (Kalueff & Cachat, 2011).

1.1.4 Reproduction

While in laboratory, zebrafish strains can breed throughout the whole year, in nature the spawning tends to be more seasonal (Spencer et al, 2008). However, there were cases of bigger females captured during January (not during the main spawning season) that had mature ova inside them, which may prove that reproduction is induced by food accessibility and not by season (Spencer et al, 2008).

In addition, sexual maturity is more likely to be linked to the individuals' size as opposed to its age and both wild and domesticated zebrafish usually hit that stage at identical sizes regardless of having different growth rates (Kalueff & Cachat, 2011).

Couples of zebrafish kept together can spawn constantly at recurrent but irregular intervals of time (Spencer et al, 2008). Studies showed that the same female can release several hundred eggs in a single spawning session, specifically 1 to 700 with a mean of 185, while

the time between sessions varies from 1 to 6 days with an average of 1.5 days (Spencer et al, 2008).

The number of eggs produced by a female is related to its body size but also with the time interval between spawning sessions (Kalueff & Cachat, 2011). This interval usually increases with the age of the female, having a mean of 1.9 days for 12-month-old females and 2.7 days for 15-month-old females (Kalueff & Cachat, 2011).

The process of ovulation requires the exposure of the female to male gonadal pheromones which is usually accomplished by placing the two together, in the same space; however, in some cases, ovulation may be induced by putting a female in the same water where the male was previously held, without requiring them to be there at the same time, because the pheromones will stay in the water (Spencer et al, 2008).

Some experiments showed that a 7-hour exposure to a male was enough for the female to lay the eggs in the next morning (Eaton & Farley, 1974). Having said that, if the female remains isolated, it is not possible to collect eggs again after that first time, which suggests that all the mature ova are liberated during the first spawning session after exposure (Eaton & Farley, 1974).

If females are isolated for too long, they may become ‘’egg-bound’’ which is a condition that causes their belly to swell due to the unreleased eggs that can become necrotic and clog the oviduct which, eventually, is lethal (Eaton & Farley, 1974).

Figure 3: Zebrafish female normal (A) and egg-bound (B) (Chopra et al, 2019). Scale bars: 1mm.

On the other hand, females exposed to males for a longer period of time before the spawning session, release more eggs with higher quality than those who were isolated and only exposed to the male the day before spawning, which can be related to the concentration of male pheromones (Kalueff & Cachat, 2011).

Zebrafish eggs usually have a diameter of about 0.7 mm; they are demersal and released directly to the substrate (Kalueff & Cachat, 2011). Before that, there is no preparation of the substrate and after the release there is no parental care by either sex (Kalueff & Cachat, 2011).

Even before contact with sperm, the unfertilized eggs begin some developmental steps, like the formation of a perivitelline space, just by exposure to the water (Schilling, 2002).

After fertilization, hatching usually occurs after 48 to 72 hours at 28.5ºC, depending on the muscular activity of the embryo and the thickness of the chorion (Schilling, 2002).

After hatching, the larvae (Figure 4) measure about 3 mm and use small secretory cells in the epidermis of the head to attach themselves to hard surfaces (Schilling, 2002). That attachment is progressively higher, which eventually lets the larvae reach the surface where they can inflate their swimming bladders, usually around 72 hours after fertilization and, after that, they begin to swim freely, to feed and perform avoidance behaviours (Schilling, 2002).

Figure 4: Zebrafish larvae. Source: (media.uzh.ch)

1.1.5 Fecundity and Fertility

Fecundity and fertility are reproductive parameters used in animal science.

When applied to fish, the fecundity rate is usually defined as the total number of eggs a female lay in one hatching (Welcomme, 1967).

However, not all of those eggs are fertilized by male sperm and therefore viable, so the number of eggs fertilized or the number of fry produced is what defines the fertility rate (Da Silva et al, 2004; Welcomme, 1967).

The concepts above can be used to evaluate zebrafish reproduction, with fecundity and fertility being assessed by the total number of eggs laid and the number of fertilized eggs respectively (Wafer et al, 2016).

1.1.6 Zebrafish as a Research Model

Zebrafish are one of the most used organisms for studying gene function in vertebrates, with their transparent embryos and genetic characteristics making it a popular model for detailed human genetic disease research (Howe et al, 2013).

Being a small vertebrate with the ability to coexist in large numbers, allows scientists to keep many individuals in a smaller space when compared to mammals (Lieschke & Currie, 2007).

In order to model zebrafish, researchers need to critically assess what results and characteristics can be extrapolated to other fish species or to all vertebrates (Segner, 2009).

About 70% of human genes have one or more zebrafish orthologue which is why they are widely used in many branches of medical studies (Lieschke & Currie, 2007).

However, using zebrafish as a model for humans is not always the best choice since it requires a higher level of abstraction than mammals, as many genes have diverged throughout time (Lieschke & Currie, 2007).

The best way to take advantage of zebrafish as a model is to study diseases in embryonic or larval states (Lieschke & Currie, 2007). Nonetheless, adults are also providing positive results in many areas and are expected to grow its use and popularity in research, although requiring a better knowledge of zebrafish anatomy and physiology (Lieschke & Currie, 2007).

The most researched area has been developmental biology. However, in the last decades, subjects like genetics and heredity, cellular and molecular biology, neurosciences and biochemistry have been increasing the research output using zebrafish (Kinth et al, 2013). The same authors conclude that it is also expected that those areas will surpass the research on developmental biology.

As for reproduction, zebrafish share many similarities with mammals, making them a popular model for infertility research (Hoo et al, 2016).

There are common mechanisms causing infertility between zebrafish and mammals, that allow us to extract information regarding human reproductive health and help find and develop better fertility medications that can be used in humans (Hoo et al, 2016).

Nowadays, the research is based on a community-minded approach, with large investments in stock facilities, open information and database tools and free trading of fish and materials (Lieschke & Currie, 2007).

However, the exponential growth in investigation is surpassing the ability of those community-based resources to provide the material necessary for all who need it (Lieschke & Currie, 2007).

In the future, zebrafish research will require greater investments in stock centres and a special focus on genome sequence programs, in order to fulfil the community's needs (Lieschke & Currie, 2007).

1.2 Environmental Enrichment

Holding facilities for research animals have often been designed to fulfil human requirements such as economic and ergonomic ones (Woodward et al, 2019). Because of that, captivity environments usually differ a lot from the animal's natural habitat, which causes major unnatural behaviours and even physiological alterations such as different blood chemistry and metabolism due to unusual stress levels (Woodward et al, 2019).

Studies have found that laboratory rats housed in enriched environments have more developed brains, more glial cells, better motor skills and improvements in the visual cortex while also showing larger behavioural diversity and complexity (Carlstead et al, 1994). These alterations improve the animal's ability to adapt its behaviour when presented with new situations or threats similarly to its behaviour in the wild (Carlstead et al, 1994).

Nowadays, to improve ethical norms in scientific research, most laboratories are adapting their facilities to mimic natural habitats creating the concept of ''Environmental Enrichment'' (Woodward et al, 2019).

The field of environmental enrichment has the goal to create captivity environments with better physical, social and temporal complexity, which allows the animals to adopt more typically wild like behaviours (Carlstead et al, 1994). The main focus is to improve the stimulation provided to the animals so that they present more natural behaviours (Woodward et al, 2019).

Environmental enrichment is usually more focused on mammals, more so on farm and domestic species, but recently, the effort has been equally invested in all types of captivity

animals (Woodward et al, 2019). Researchers today are legally obligated to improve and consider animal welfare, including fish (Lee, 2017).

Regarding fish species, it is difficult to define their natural or normal habits since there is not much information on how to induce a stress-free and normal behaviour (Woodward et al, 2019). However, environmental enrichment has been deeply studied when applied to boost populations of commercially reared species (Woodward et al, 2019).

Environmental enrichment for research and commercial fish usually implies improving the tank's structural complexity by introducing objects or plants that mimic their natural habitat (Woodward et al, 2019). This includes gravel, stones, plastic tubes, artificial and real plants, driftwood and plant pots or even adding colourful lights (Woodward et al, 2019). For example, one investigation showed that adding gravel and plants to zebrafish tanks improved their survivorship and growth (Lee, 2017).

Some studies on zebrafish revealed that fish housed in enriched environments showed less aggressive interactions, more cautious behaviour and faster reaction times when compared to fish housed in empty tanks (Woodward et al, 2019).

Adding artificial plants that mimic the natural conditions to a tank may serve as shelter and provide refuge and safes spaces for zebrafish which allows them to prevent negative social encounters and also protects them from artificial lights or water flow that may cause disturbance (Stevens et al, 2021).
At the same time, substrate can help with camouflage which provides the sensation of safety and therefore the genera welfare (Stevens et al, 2021)

Regarding beneficial impacts on commercial species, such as in the body size, the results are inconclusive since some studies report bigger individuals when housed in enriched tanks, but other studies show the contrary (Woodward et al, 2019).

1.3 Environmental Enrichment and Reproduction

Regarding the effects of environmental enrichment in reproduction, decades ago, scientists found a ‘’sterility factor’’ in captivity animals that reduced their reproduction rates, which was most likely caused by poor and simple housing conditions (Carlstead et al, 1994). Until 1951 there were no successful births of species like Indian rhino (*Rhinoceros unicornis)*, okapi *(Okapia johnstoni)*, gorillas *(Gorilla gorilla)*, cheetahs *(Acinonyx jubatus)* and flamingos *(Phoenicopterus ruber)* kept in captivity (Carlstead et al, 1994).

However, by the 60’s, many zoos had accomplished to reproduce these species after realizing the importance and influence that external conditions had on reproductive habits and, therefore, creating enriched nature-like environments for them (Carlstead et al, 1994). Nowadays, we are aware of the importance of enriched environments regarding nutrition, climate, substrates, housing, nesting and social interactions to induce normal reproductive patterns (Woodward et al, 2019).

Many studies have been conducted to determine how the environment influences the reproductive outcome through physiological, psychological and sociological processes (Woodward et al, 2019). However, in the case of zebrafish, there are many different results.

In 2007, a study found that enriched tanks with substrate were correlated to higher egg survival rates until hatching, but enriched tanks with vegetation showed no significant difference from control tanks (Spence et al, 2007). In line with the latter finding, in 2009, a study concluded that enriched tanks with artificial plants showed no significant difference in zebrafish fecundity rates (Carfagnini et al, 2009).

More recently, Wafer et al (2016) concluded that environmental enrichment could improve zebrafish fertility and fecundity under certain conditions. Zebrafish housed in tanks with artificial grass had greater egg production and higher fertility and fecundity rates than those housed with artificial leaves or bare tanks (Wafer et al, 2016).

However, a study in 2018 found no significant differences in reproductive outputs between fish housed in enriched tanks and fish housed in bare tanks (Lee et al, 2018).

After that, in 2019, a study found that zebrafish females produced 54% more eggs in mildly enhanced tanks than in control tanks (Woodward et al, 2019). However, very enhanced

tanks showed a decrease in egg production when compared to mildly enhanced tanks but no significant difference to control tanks. The same study also concluded that neither level of enhancement influenced fertility rates.

To this day, studies regarding the effect of environmental enrichment on zebrafish reproduction have no singular conclusion because no consistent correlation has been found between the two.

In addition, in the few cases in which this correlation exists, it depends and varies much between the different enrichment methods. These deserve more studies and refinement.

1.4 Study Aims and Goals

As mentioned before, there is ample evidence that environmental enrichment improves animal welfare overall. However, regarding fish, there is no definite conclusion if enrichment improves reproduction and, if so, how.

Therefore, the aim of this study is to contribute to determining the influence of environmental enrichment on zebrafish fertility and fecundity. More specifically, this study's goals are to investigate if the presence of gravel with plastic plants, and its exposure time, influence:

1. the fecundity rate;
2. the fertility rate;
3. and egg survival until hatching.

2. Material and Methods

2.1 Zebrafish and overall design

In this study, 36 adult zebrafish were used for the entire timeline. The animals were a pet store-purchased (PET) wild-type (WT) strain, as per the terminology of Audira et al (2020).

For the experiments, they were randomly divided into 3 groups of 12 (G1, G2 and G3), each one subdivided into 2 groups of 6 (treated and control). The treated and control groups contained 4 females and 2 males each. The design is displayed in Figure 5.

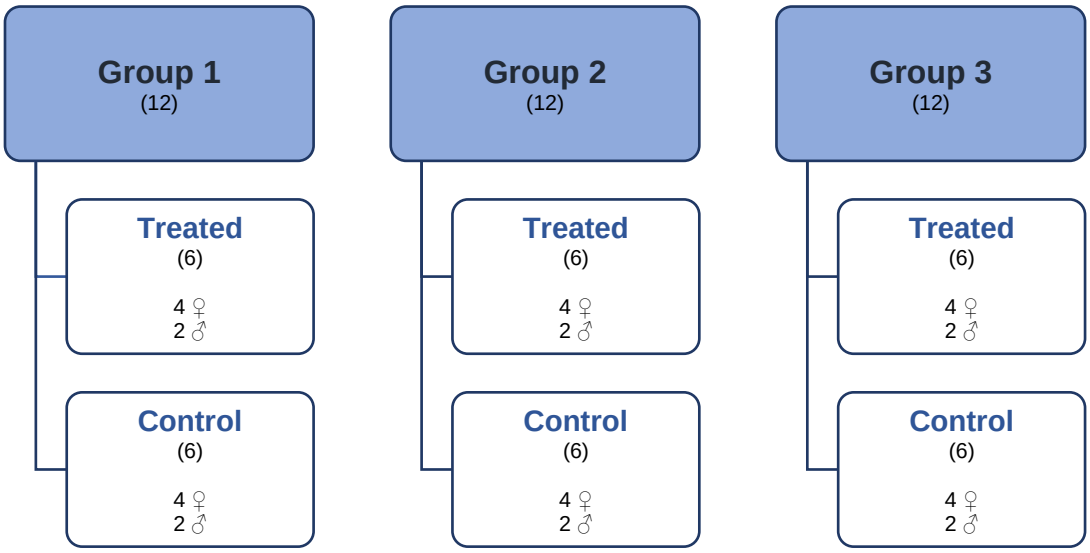

Figure 5: Distribution and number of fish per group.

Each group was weighted in the last week of the study so that the results could be adjusted to female weight. The weighting process was made using a digital balance and by placing the fish one by one in a plastic beaker on top of the balance.

2.2 Housing

Each fish group was housed in 20 cm x 20 cm x 20 cm glass tanks, with 5 L of dechlorinated tap water each, in a recirculating water system in which all tanks were connected to a filtration centre (Figure 6). The tanks were cleaned from debris once a week using a syphon.

The temperature was set at 28 ± 1ºC and measured daily to ensure stability. Each tank contained one heater and one aeration tube to promote the oxygenation of the water.

The tanks were in a room with controlled automated lighting turning on at 7 AM and off at 7 PM, providing a cycle of 12h light and 12h dark.

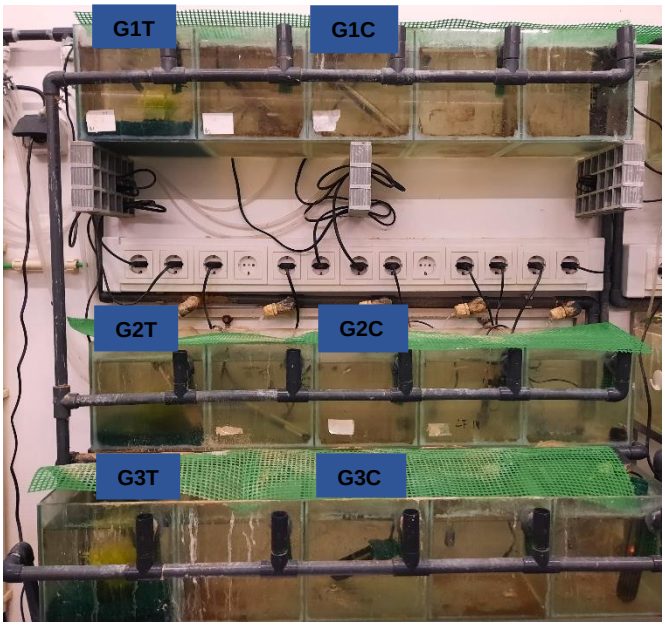

Figure 6: Tank system used in this study and fish group distribution. G1T= Group 1 Treated; G1C = Group 1 Control; G2T = Group 2 Treated; G2C = Group 2 Control; G3T = Group 3 Treated; G3C = Group 3 Control.

Water quality assessments (pH, ammonia, nitrite and nitrate) were made monthly with commercial kits to ensure all parameters were safe and within guidelines for zebrafish:
PH: between 6.8 and 7.5; Ammonia <0.02 mg/L; Nitrate <50 mg/L; Nitrite <0.1 mg/L (Avdesh et al, 2012).

2.3 Feeding

Fish were fed 3 times a day, 2 times with commercial flakes (TetraMin Tropical) and 1 time with frozen *Artemia spp*. nauplii (Ocean Nutrition). The quantities were provided so that consumption would end in up to 1 min.

2.4 Environmental Enrichment Exposure

The treated groups were exposed to environmental enrichment for different periods of time. During that exposure, those groups were housed in tanks decorated with 3 cm of gravel (2-4 mm) and two 10 cm high plastic plants, in addition to the material described above (Figure 7).

Figure 7: Enriched tanks, each with two plastic (centrally positioned) plants on the top of gravel as sediment.

There were two different periods of environmental enrichment exposure tested in this study: 7 and 14 days.

To that end, all 6 fish in each treated groups were transferred together to the enriched tanks, before being put in the reproduction tank.

At the same time, control groups were transferred to bare tanks to ensure equal conditions and stress levels that could occur with moving and handling.

Outside the exposure periods, both treated and control groups were housed in bare tanks with only the basic equipment mentioned above.

Exposures of 7 and 14 days were repeated 3 times (A, B and C) for each group, summing 18 trials (6 for each group).

2.5 Reproduction

The day before the morning of reproduction, usually around 5 pm, each group (G1, G2 or G3) was transferred to a reproduction tank. That tank was made with a larger plastic container filled with dechlorinated tap water kept at 28 ± 1ºC with a heater. Inside the tank, there were two smaller plastic containers, one for the treated and one for the control group.

Those smaller recipients contained a net (creating a separated bottom in which the eggs could fall and not be eaten by adults), glass marbles to simulate the natural habitat (Krueger et al, 2020), and one aeration tube.

The reproduction tank was isolated from the light of the room and had an automated lamp, which also provided a 12 h light cycle, turning On and Off at 7 am and 7pm respectively. At 7 am, with the light turning on, oviposition was induced.

The fish were kept in the reproduction tank from 5 pm of the day before to 9 am of the day of oviposition. Progenitors were then returned to bare tanks until it was time to initiate a new exposure, one week later.

2.6 Egg Collection and Counting

At 9 am, 2 hours after oviposition, the eggs were collected using a plastic pipette with the end cut off. Each egg was gently transferred to a glass beaker. The unviable eggs, identified by their white opaque colour, were counted and disposed.

After all eggs were collected and the unviable one were disposed of, the viable ones were washed 3 times using water from the system. The glass beakers were then identified and placed in the bigger recipient of the reproduction tank to maintain temperature.

The eggs were analysed and counted and unviable ones removed every 24h until hatching; from 0 h post fertilization (pf) to 72 h pf.

Using the total number of eggs at oviposition it was possible to access the fecundity rate, and using the percentage of viable eggs it was possible to access the fertility rate.

To adjust the results to the female fish weight, the total number of eggs at 0 h pf was divided by the mean of the group's female weight.

Also, by counting the eggs every 24 h it was also possible to access whether and how the survival rate was affected.

2.7 Data Analysis

Inferential analyses were made using IBM SPSS Statistics 27 software.
All data was pre-examined for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test).
Then, data regarding the number of eggs produced and also the number of viable eggs was analysed with a one-way analysis of variance (ANOVA), with the exposure to environmental enrrichment as the fixed factor. The same test was then applied, individually, for the different exposure periods of time.
The significance level (α) was set at 0.05, and if $p < \alpha$ it was accepted as statistically significant.

3. Results

3.1 Fish Weight and Raw Numbers from Egg Counting

Table 1 depicts the means of female and male weight for each group so that the number of eggs can be adjusted to the mean of female weight for each group.

Group 1 has a weight mean of 1.22g (SD=0.51) for Control and 1.57g (SD=0.41) for Treated.

Group 2 has a weight mean of 1.25g (SD=0.15) for Control and 1.50g (SD=0.27) for Treated.

Group 3 has a weight mean of 1.40g (SD=0.45) for Control and 1.51g (SD=0.56) for Treated.

Table 1: Fish weight (g) and mean.

Group	Fish	Weight (g)	Mean
1 Control	♀1	1.27	♀: 1.44g ♂: 0.77g
	♀2	1.15	
	♀3	1.10	
	♀4	2.25	
	♂1	0.90	
	♂2	0.63	
1 Treated	♀1	1.20	♀: 1.49g ♂: 1.73g
	♀2	2.15	
	♀3	1.49	
	♀4	1.13	
	♂1	2.10	
	♂2	1.35	
2 Control	♀1	1.31	♀: 1.31g ♂: 1.13g
	♀2	1.38	
	♀3	1.15	
	♀4	1.41	
	♂1	0.98	
	♂2	1.27	
2 Treated	♀1	1.10	♀: 1.48g ♂: 1.53g
	♀2	1.25	
	♀3	1.81	
	♀4	1.76	
	♂1	1.41	
	♂2	1.64	
3 Control	♀1	2.03	♀: 1.36g ♂: 1.45g
	♀2	1.38	
	♀3	0.73	
	♀4	1.31	
	♂1	1.88	
	♂2	1.02	
3 Treated	♀1	0.73	♀: 1.42g ♂: 1.68g
	♀2	1.62	
	♀3	2.01	
	♀4	1.32	
	♂1	1.01	
	♂2	2.35	

Table 2: Egg counting results. (V=Viable; U=Unviable)

Exposure	Group		Eggs							
			0 h pf		24 h pf		48 h pf		72 h pf	
			V	U	V	U	V	U	V	U
7 days A	1	Control	200	20	100	100	80	20	/	/
		Treated	270	10	160	110	120	40	/	/
	2	Control	340	20	235	105	120	115	75	45
		Treated	160	40	110	50	55	55	35	20
	3	Control	97	5	95	2	90	5	85	5
		Treated	305	10	300	5	290	10	270	20
7 days B	1	Control	150	10	100	50	70	30	50	20
		Treated	485	15	350	135	330	20	300	30
	2	Control	450	30	380	70	330	50	290	40
		Treated	200	50	180	20	170	10	150	20
	3	Control	107	3	100	7	95	5	90	5
		Treated	330	5	320	10	300	20	295	5
7 days C	1	Control	250	15	200	50	140	60	100	40
		Treated	120	10	115	5	100	15	100	0
	2	Control	190	50	175	15	160	15	150	10
		Treated	250	20	240	10	215	25	200	15
	3	Control	150	10	140	10	125	15	120	5
		Treated	380	15	375	5	360	15	350	10
14 days A	1	Control	450	20	380	70	320	60	280	40
		Treated	250	30	200	50	190	10	170	20
	2	Control	450	30	350	100	310	40	300	10
		Treated	500	15	430	70	405	25	400	5
	3	Control	150	10	145	5	135	10	120	15
		Treated	340	35	330	10	325	5	315	10
14 days B	1	Control	300	20	280	20	250	30	200	50
		Treated	275	10	270	5	260	10	250	10
	2	Control	525	30	500	25	450	50	400	50
		Treated	175	10	140	35	100	40	90	10
	3	Control	170	10	160	10	145	15	140	5
		Treated	330	5	325	5	315	10	315	0
14 days C	1	Control	250	5	200	50	150	50	100	50
		Treated	200	10	180	20	170	10	150	20
	2	Control	530	20	450	80	400	50	300	100
		Treated	200	10	200	0	190	10	179	11
	3	Control	105	10	100	5	80	20	70	10
		Treated	320	5	315	5	300	15	285	15

In Table 2 the results show the number of eggs counted in all groups at different times after oviposition, as well as the difference between viable and unviable ones.

It was found that the mean number of total eggs produced at oviposition was 288 (SD=157) for control groups and 300 (SD=102) for treated groups.

However, the mean percentage of viable eggs at oviposition was the same (94%) for both control and treated groups.

Table 3: Egg counting results adjusted to female weight.

Exposure	Group		Total Eggs at 0h pf	Total Eggs at 0h pf divided by the respective group's female weight mean (in grams)
7 Days A	1	Control	220	153
		Treated	280	188
	2	Control	360	275
		Treated	200	135
	3	Control	102	75
		Treated	315	222
7 Days B	1	Control	160	111
		Treated	500	336
	2	Control	480	367
		Treated	250	169
	3	Control	110	81
		Treated	335	236
7 Days C	1	Control	265	184
		Treated	130	87
	2	Control	240	183
		Treated	270	182
	3	Control	160	118
		Treated	395	278
14 Days A	1	Control	470	326
		Treated	280	188
	2	Control	320	222
		Treated	285	191
	3	Control	160	118
		Treated	375	264
14 Days B	1	Control	320	222
		Treated	285	191
	2	Control	555	424
		Treated	185	125
	3	Control	180	132
		Treated	335	236
14 Days C	1	Control	255	177
		Treated	210	141
	2	Control	550	424
		Treated	210	142
	3	Control	115	85
		Treated	325	229

When results were adjusted to female weight, the mean number of eggs at oviposition was 212 (SD=121) for control groups and 205 (SD=71) for treated groups (Table 3).

3.2 Fecundity

On a first analyses it is possible to see, as shown in Table 4, that the mean of total eggs at 0h pf appears to be higher for the treated groups. However, when the number of total eggs is adjusted to the mean of the group's female weight, the mean of total eggs at 0h pf appears to be higher for control groups.

Table 4: Total number of eggs at 0h pf and of total number of eggs per female mean weight at 0h pf.

Exposure		Total Eggs at 0h pf	Eggs per Mean Female Weight at 0h pf
Control	Mean	288	212
	N	18	18
	Std. Deviation	157	121
Treated	Mean	300	205
	N	18	18
	Std. Deviation	102	71
Total	Mean	294	209
	N	36	36
	Std. Deviation	131	97

The null hypothesis tested by ANOVA was that there are no significant differences between the means of total eggs, adjusted to the mean female weight at 0h pf for control and treated groups (Table 4).

Table 5: ANOVA results: mean number of eggs at 0h pf adjusted to female weight for control and treated groups.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	419.165	1	419.165	0.043	0.837
WITHIN GROUPS	332195.607	34	9770.459		
TOTAL	332614.772	35			

As the results show, there is no evidence to reject the hypothesis (p = 0.837), meaning that the difference is statistically insignificant.

Regarding the exposure time influence, as seen in Figure 6, it seems that bigger exposure times had no evident influence in the number of eggs at 0h pf for treated groups.

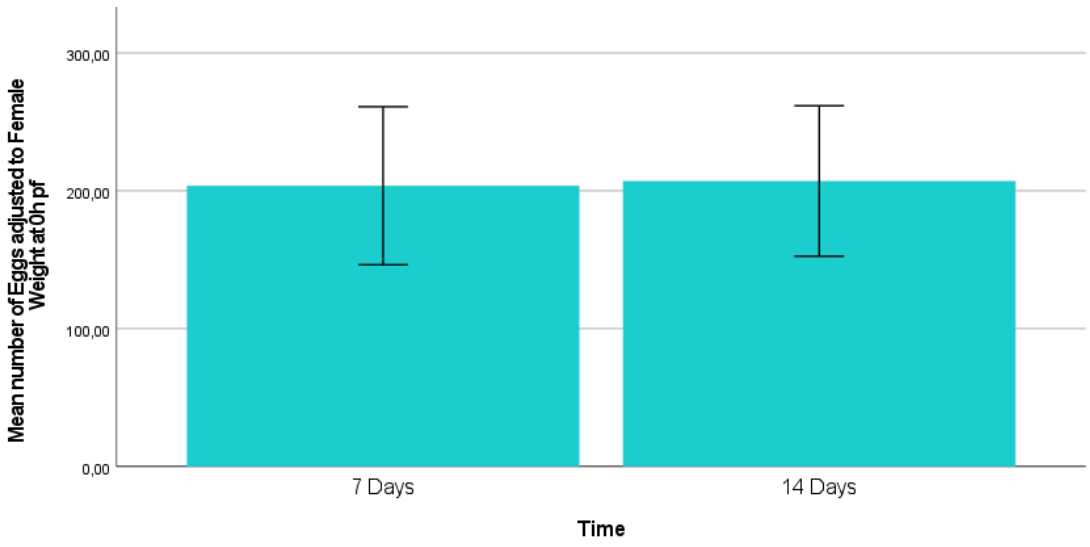

Figure 8: Mean number of eggs (N=9) adjusted to female mean weight at 0h pf for treated groups by exposure time.

Table 6: Total number of eggs adjusted to female weight at 0h pf for treated groups by exposure time.

Exposure Time	Mean	N	Std. Deviation
7 Days	204	9	75
14 Days	207	9	71
Total	205	18	71

A One-Way ANOVA was run to test the significance of those differences with the null hypothesis saying that there are no significant differences between the mean number of total eggs adjusted to female weight at 0h pf for treated groups at different exposure times.

Table 7: ANOVA results: mean number of eggs at 0h pf adjusted to female weight for treated groups by exposure time.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	52.429	1	52.429	0.010	0.922
WITHIN GROUPS	84858.093	16	5303.631		
TOTAL	84910.522	17			

As the results show (Table 7), there is no evidence to reject the hypothesis (p=0.922), meaning that the difference is statistically insignificant.

3.3 Fertility Rate

On a first analysis (Table 8), it was possible to see that the mean percentage of viable eggs at 0h pf was the same for control and treated groups. However, ANOVA was obviously not significant (Table 9).

Table 8: Mean and Std. Deviation of percentage (%) of viable eggs at 0h pf.

Exposure	Mean	N	Std. Deviation
Control	93.6	18	4.0
Treated	93.7	18	5.6
Total	93.6	36	4.8

Table 9: ANOVA results: percentage of viable eggs at 0h pf for control and treated groups.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	0.41	1	0.041	0.002	0.967
WITHIN GROUPS	806.803	34	23.730		
TOTAL	806.844	35			

As the results show, there is no evidence to reject the hypothesis (p=0.967), meaning that the difference is statistically insignificant.

If different exposure times are considered (Table 10), the results stay similar, showing an apparently small increase in the percentage of viable eggs of treated groups for 14 days when compared to treated groups for 7 days.

Table 10: Mean and Std. Deviation of percentage of viable eggs at 0h pf for treated groups by exposure time.

Exposure Time	Mean	N	Std. Deviation
7 Days	92.2	9	7.2
14 Days	95.1	9	3.2
Total	93.6	18	5.6

ANOVA was run to test the significance of the differences, with a null hypothesis saying that there are no significant differences between the mean percentage of viable eggs at 0h pf for treated groups at different exposure times.

Table 11: ANOVA results: percentage of viable eggs at 0h pf for treated groups by exposure time.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	36.713	1	36.713	1.178	0.294
WITHIN GROUPS	498.503	16	31.156		
TOTAL	535.215	17			

As the results show (Table 11), there is no evidence to reject the hypothesis (p=0,294), meaning that the difference is statistically insignificant.

3.4 Egg survival

On a first analysis, comparing the mean percentage of egg survival at 24h pf, 48h pf, and 72h pf between control and treated groups, it seems there is a slight increase for the treated groups (Table 12).

Table 12: Percentage of egg survival at 24h pf, 48h pf and 72h pf for control and treated groups.

Exposure		Percentage of Viable Eggs at 24h pf	Percentage of Viable Eggs at 48h pf	Percentage of Viable Eggs at 72h pf
Control	**Mean**	84.9	83.8	84.8
	N	18	18	17
	Std. Deviation	12.8	11.3	11.3
Treated	**Mean**	89.1	89.6	92.5
	N	18	18	17
	Std. Deviation	12.2	12.3	8.4
Total	**Mean**	87.0	86.7	88.6
	N	36	36	34
	Std. Deviation	12.5	12.0	10.6

In addition, it seems that the percentage of survival slightly declined from 24h pf to 48h pf, going up again at 72h pf, for both control and treated groups (Figure 9).

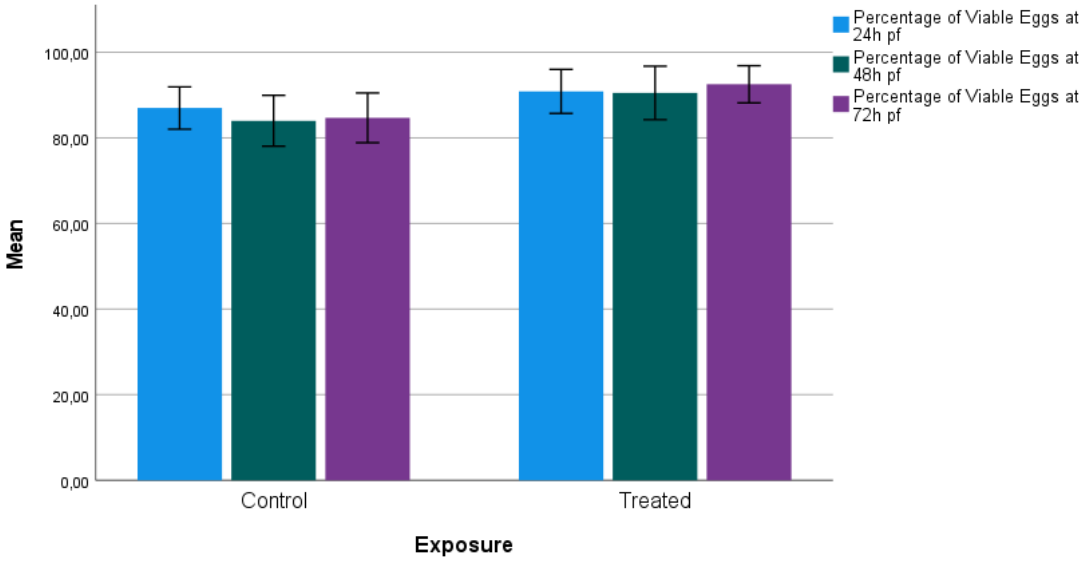

Figure 9: Mean percentage and SD of egg survival at 24h, 48h and 72 pf for control and treated groups.

A One-Way ANOVA was run for data at 24h and again for data at 48h and 72h pf, to test the significance of the differences between controls and treated at each time point.

The null hypothesis says that there are no significant differences between the mean percentage of viable eggs at 24h, 48h and 72h pf between control and treated groups.

Table 13: ANOVA results: mean percentage of egg survival at 24h pf for control and treated groups.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	157.921	1	157.921	1.010	0.322
WITHIN GROUPS	5313.874	34	156.290		
TOTAL	5471.795	35			

For 24h pf, as the results show a p value of 0.322 (Table 13), there is no evidence to reject the hypothesis, meaning that the difference is statistically insignificant.

Table 14: ANOVA results: mean percentage of egg survival at 48h pf for control and treated groups.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	310.030	1	310.030	2.226	0.145
WITHIN GROUPS	4735.974	34	139.293		
TOTAL	5046.003	35			

For 48h pf, as the results shown a p value of 0.145 (Table 14), there is no evidence to reject the hypothesis, meaning that the difference is statistically insignificant.

Table 15: ANOVA results: mean percentage of egg survival at 72h pf for control and treated groups.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	522.515	1	522.515	5.263	0.028
WITHIN GROUPS	3177.100	32	99.284		
TOTAL	3699.615	33			

For 72h pf, as the results show a p-value of 0.028 (Table 15), it is possible to reject the hypothesis, meaning that the difference is statistically significant.

If different exposures times are considered (Table 16 and Figure 8), the results suggest an increased percentage of egg survival in treated groups for 14 days when compared to treated groups for 7 days, for 24h, 48h and 72h pf.

Table 16: Percentage of egg survival at 24h, 48h and 72h pf for treated groups by exposure time.

Time		Percentage of Viable Eggs at 24h pf	Percentage of Viable Eggs at 48h pf	Percentage of viable eggs at 72h pf
7 Days	Mean	86.2	86.3	90.6
	N	9	9	8
	Std. Deviation	15.2	15.2	11.6
14 Days	Mean	92.0	93	94.3
	N	9	9	9
	Std. Deviation	8.2	8.2	4.2
Total	Mean	89.1	89.6	92.5
	N	18	18	17
	Std. Deviation	12.2	12.3	8.4

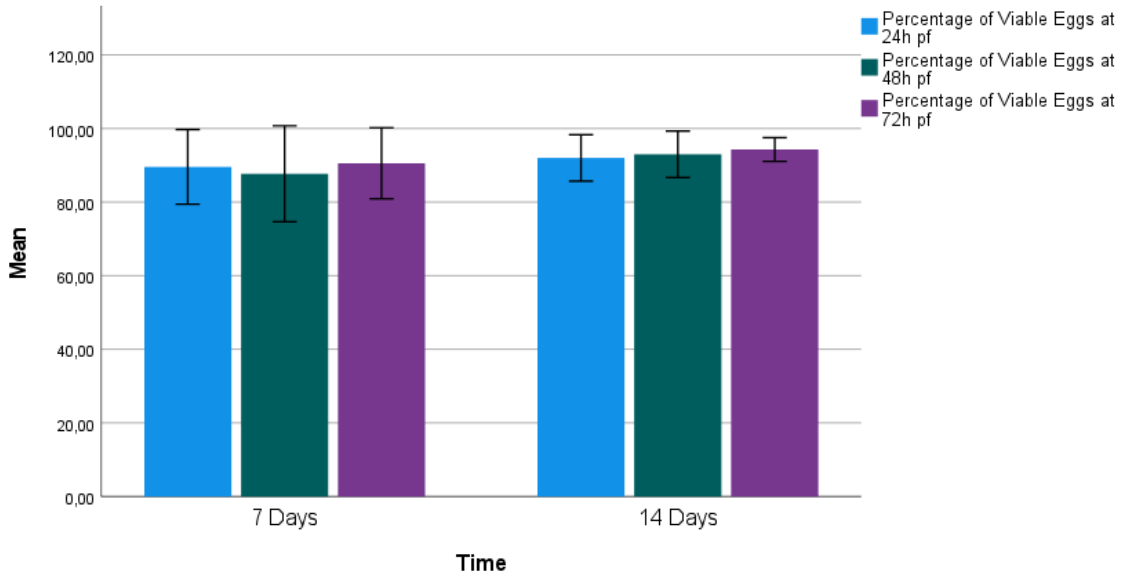

Figure 10: Mean percentage and SD of egg survival at 24h, 48h and 72h pf for treated groups by exposure time.

A One-Way ANOVA was run individually for data at 24h, 48h and 72h pf, to test the significance of the differences.

The null hypothesis says that there are no significant differences between the mean percentage of viable eggs at 24h, 48h and 72h pf for treated groups at different exposure times.

Table 17: ANOVA results: mean percentage of egg survival at 24h pf for treated groups by exposure time.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	151.037	1	151.037	1.011	0.330
WITHIN GROUPS	2389.586	16	149.349		
TOTAL	2540.623	17			

For 24h pf, as the results shown a p value of 0.330 (Table 17), there is no evidence to reject the hypothesis, meaning that the difference is statistically insignificant.

Table 18: ANOVA results: mean percentage of egg survival at 48h pf for treated groups by exposure time.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	202.103	1	202.103	1.358	0.261
WITHIN GROUPS	2381.924	16	148.870		
TOTAL	2584.027	17			

For 48h pf, as the results shown a p value of 0.261 (Table 18), there is no evidence to reject the hypothesis, meaning that the difference is statistically insignificant.

Table 19: ANOVA results: mean percentage of egg survival at 72h pf for treated groups by exposure time.

	SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.
BETWEEN GROUPS	59.527	1	59.527	0.828	0.377

WITHIN GROUPS	1078.677	15	71.912
TOTAL	1138.204	16	

For 72h pf, as the results shown a p value of 0.377 (Table 19), there is no evidence to reject the hypothesis, meaning that the difference is statistically insignificant.

4. Discussion

There have been many studies conducted to determine how the environment influences the reproductive outcome of animals (Woodward et al, 2019). However, regarding zebrafish, results have been inconclusive. Some found that environmental enrichment could improve zebrafish fertility and fecundity (Wafer et al, 2016), but others found no significant differences in reproductive outcome (Lee et al, 2018; Carfagnini et al, 2009).

In this study, to further explore this subject, it was tested if environmental enrichment in the form of plants and gravel influenced fecundity and fertility rates and egg survival until hatching.

Regarding fecundity, this study found that the mean number of eggs produced per gram of average female weight did not differ between treated and control groups.
This result is consistent with previous studies with zebrafish that found no correlation between reproductive outcome and environmental enrichment in the form of gravel and two types of plants (Lee et al, 2018; Carfagnini et al, 2009), suggesting that neither of those elements has an impact in zebrafish fecundity.

Still regarding fecundity, when different time exposures were considered, at first glance, it seemed that there was a slight increase in the number of eggs produced by gram of female weight in groups exposed for 14 days, compared to groups exposed for 7 days. However, this difference was not statistically significant. This result is also concordant with previous studies with zebrafish and the same type of enrichment (Lee et al, 2018; Carfagnini et al, 2009) in the sense that environmental enrichment did not increase the fecundity rate. However, few have tested if different time exposures would have other effects.

Concerning fertility, the results were similar. There was no apparent or significant difference between the percentages of viable eggs for treated and control groups. These results are consistent with other previous studies with zebrafish, that also found no correlation between environmental enrichment in the form of gravel and plants (Lee et al, 2018) and in the form of plants and decorative pots and seascape design (Woodward et al, 2019) and fertility rates, suggesting that neither of those enrichment tools had any influence in the fertility of zebrafish.

If considering different time exposures, there was a suggestive increase in the percentage of viable eggs in groups treated for 14 days compared to groups treated for 7 days.

However, this difference was also not statistically significant, which is expected since there is not much previous information about the correlation between environmental enrichment and fertility.

Finally, regarding egg survival, there was a slight increase in the percentage of viable eggs at 24h pf, 48h pf and 72h pf in treated groups. Unlike the differences at 24h pf and 48h pf, which were not significant, the increase in viable eggs at 72h pf for treated groups showed statistically significant results.

The latter result concords with previous studies that found significant differences in egg survival between environmental enriched and control groups (Spence et al, 2007; Lee et al, 2017), using the combination of plants and gravel as the form of environmental enrichment. However, those differences were found at different periods of time post fertilization: 5 to 30 days pf (Lee et al, 2018) and only 3 days pf (Spence et al, 2007). In the present study, the increase in survival only started at 72h pf (3 days pf) which aligns with the results of the studies mentioned above that also only started at 3 days pf.

This result may be explained considering the evidence that environmental enrichment improves welfare and decreases the stress levels which ultimately leads to better general health that may improve the quality of the eggs produced (Stevens et al, 2021).

On the other hand, the fact that at 24h pf and 48h pf there was no significant difference in survival between enriched and control groups is also concordant with previous studies that found no correlation between environmental enrichment, in the form of plants and grass, and egg survival at 6 days pf (Wafer et al, 2016). This opposition of results suggest that other factors may be involved when the survival rate increases.

If different exposure times were taken into consideration, there was a suggestive increase in percentage of viable eggs for groups treated for 14 days comparing to groups treated for 7 days, for 24h pf, 48h pf, and 72h pf. Even so, these differences showed no statistical significance, which could be expected since there was no previous research found about how different times of environmental enrichment exposure could impact egg survival.

5. Conclusion

The environmental enrichment tested in this study had no significant influence in zebrafish fecundity, fertility, or egg survival, even if trying two exposure times (7 and 14 days).

However, the results show that groups of breeders exposed to environmental enrichment produced eggs with a higher survival rate at 72h pf than those produced by control groups.

Overall, the study suggests that maybe a simple container with good quality water and enough feeding, and an optimal sex ratio, is good enough for promoting good breeding in adult zebrafish in the laboratory. If that is the case, this simplifies the handling by researchers.

Yet, it is vital to notice that environmental enrichment encompasses many different types of objects, materials, substrates, and plants arranged in countless combinations and positions.

This study only tested one form of environmental enrichment made with gravel and plants. Therefore, the results cannot be extrapolated to all forms of environmental enrichment.

In the end, this study's data can be used as a base to further investigate the influences of environmental enrichment in multiple parameters of reproductive outcomes for zebrafish.

6. Future perspectives

Although environmental enrichment and its effects on reproductive outcomes on zebrafish are becoming an increasingly studied topic, there is not yet a universal and robust conclusion on the matter since results diverge a lot. Accordingly, and given the number of variables, there is a need for further studies to continue to test this possible correlation.

More forms of environmental enrichment need to be tested. The concept provides infinite possibilities due to all the different materials that should be put to trial in several different combinations, durations and positions.

Also, while this study focused on exposing groups of breeders to environmental enrichment, future studies should test exposures to only females, only males, only during oviposition, or even only to the eggs or fry after oviposition, since those may provide different results.

It is also pertinent to prolong the survival tests even longer to see the influence that enrichment might have on the survival and development of zebrafish fry until the adult size.

To summarize, environmental enrichment and its effect on zebrafish reproductive outcomes is a complex subject with innumerous possibilities and potential for further discoveries.

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