Hypergravity induces changes in physiology, gene expression and epigenetics in zebrafish

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Abstract

All living organisms that inhabit Earth have evolved under a common value of gravity, which amounts to an acceleration of 9.81 m/s^2 at mean sea level. Changes on it could cause important alterations that affect vital biological functions. The crescent interest in spatial exploration has opened the question of how exactly these changes in gravity would affect Earth life forms on space environments. This work is the result of a collaborative co-supervision of a master thesis between experts in the area of space sciences and biology, and it can serve as a case study for training experts in such interdisciplinary environments. In particular, we focus on the effect of gravity as a pressure factor in the development of zebrafish (*Danio rerio*) in the larval stage as a model organism using up-to-date (genomic and epigenetic) techniques. Given the high cost of any experiment in true low gravity (which would require a space launch), we performed an initial experiment in hypergravity to develop the methodologies and identify good (epi)genetic markers of the effect of gravity in our model organism. Previous studies in zebrafish have shown how alteration in gravity effects the development and the gene expression of important regulatory genes. For this study, we firstly customized a small laboratory scale centrifuge to study changes in fish physiology together with changes at molecular levels. We exposed zebrafish larvae from 0 to 6 days post fertilization to the simulated hypergravity (SHG) (100 rpm ~ 3g). After 6 days of hypergravity exposition the larvae showed changes in their swimming and flotation patterns, and presented corporal alterations. Then, we assessed gene expression of genes implicated in important biological processes, (e.g., epigenetics), and an upregulation were observed when compared to the control. Taken together, these preliminary findings show how gravity alterations could affect some basic biological responses, and illustrate the potential of developing new science cases to be developed by students at postgraduate level (MSc and beyond) in a multidisciplinary environment.

Keywords

Biology, Behavior, Gravity, Gene Expression, Multidisciplinary, Space Environment

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Abbreviations

Dpf *Days post fertilization*

Hpf *Hours post fertilization*

SHG *Simulated Hypergravity*

1. Introduction

All the living organisms that inhabit the Earth have evolved under the same value of gravity, which amounts to an acceleration of 9.81 m/s2 at mean sea level [1]. Gravity has been responsible to shaping the life forms, its organs, bodies, and for consequence is involved in its behaviour [2], [3]. Currently we can find a few of studies that both simulate changes in gravity or study directly in the space, however, implementing these is not easy. Instead, a used tool to analyse the organisms changes under a different gravity is Simulated Hypergravity (SHG), where a centrifuge machine is used to simulate a gravity excess, that is, an acceleration greater than 9.81 m/s^2.

Studies under natural or simulated altered gravity have shown how the change on it is able to alter different systems on animal, cellular and human models. An investigation performed by Fritsch-Yelle and co-workers [4], shown how the arterial pressure and cardiac rhythm of 12 astronauts during a space missions were altered and decreased with respect to their normal values; and hypergravity also affected important systems such as, musculoskeletal system and bone formation in zebrafish [5], [6]. Zebrafish (*Danio rerio*) has been used on gravity altered studies, because it is an important animal model, that has been successfully used for a lot of scientific researches for more than 20 years thanks to its convenient features, such as: short generation time, high amount of eggs produced by each mating, and high number of orthologous genes with humans [7].

Epigenetics are defined as alterations in the gene function that do not involve changes in the nucleotide changes in the DNA. DNA methylation is a type of epigenetic event that implies modifications an addition of a -CH3 group at cytosines, primarily in CpG sites [8], [9]. DNA- methyltransferase 1 and 3 (*dnmt1, dnmt3*) and the tet methylcytosine dioxygenase 1 (*tet1)* are genes involved in epigenetic events, and thus considered as good epimarkers.

Given previous studies, we hypothesize that the alteration of gravity (hypergravity) will also have effects on the physiology, behaviour and gene expression of genes involved in DNA methylation mechanisms in zebrafish, and these effects can be measured on epigenetic markers. Developing the methodology to study these gravity sensitive markers for future studies in a space environment is the aim of this work

2. Materials and Methods

2.1. Centrifuge

To simulate the hypergravity we use a rotary mixer machine with a maximum speed of 100 rpm (model: ANR100DE, OVAN laboratory equipment), and added two perpendicular arms of 25 cm of long ended in two gondolas with the capacity to contain the plates with the zebrafish larvae (Figure 1).

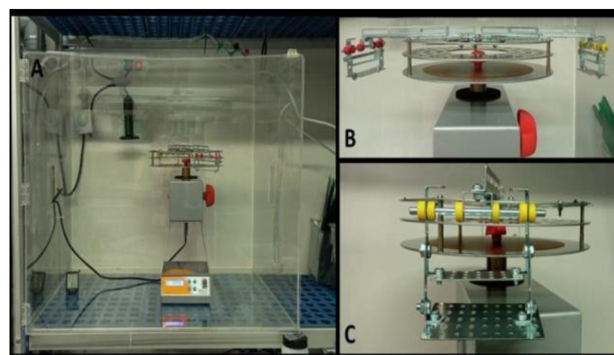

Figure 1. A: Incubator and centrifuge, B: Arms with gondolas, C: Gondola.

2.2. Zebrafish larvae

Zebrafish (TUE strain) were housed in the animal facilities of the experimental aquariums zone (ZAE) at the Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). Fish were held in 10L tanks on a recirculating system (Aquaneering, San Diego, CA) with a water pump of 3000 L/h and a UV light system to eliminate any possible bacteria in the water (Figure 5A) with a 12:12 h light: dark cycle. Water was maintained at (28 ± 0.2°C), pH 12 (7.2 ± 0.5), conductivity (750–900 µS) and dissolved oxygen (6.5–7.0 mg/l) [4], and monitored daily. Fish were fed twice daily, receiving dried food and live Artemia nauplii (AF48, INVE Aquaculture, Dendermonde, Belgium). The experiment was approved by CSIC ethical committee with the number 1166/2021.

2.3. Experimental conditions

A total of 4 independent breeding pairs were used. Once the eggs were fertilized they were collected and putted in the plates and tubes with 250 µL of embryo medium, and covered with adhesive sealer to avoid fluid loss, then they were placed into the gondolas to start the experimentation. At the same time controls were placed next to the centrifuge machine, and

they were maintained at 27- 28°C, with 60-65% humidity and the temperature was measured and controlled daily.

The total number of analyzed individuals was 440 for the SHG condition, and 220 for Control, divided in four biological replicates. The centrifugation protocol was set to 100 rpm (revolutions per minute) spin delivering a centripetal acceleration (ac) of 25.8 m/s^2 that vectorially added to the existing Earth acceleration (g) of 9.8 m/s^2 , it results in an acceleration of 27.6 m/s2 ($a_T = \sqrt{g^2 + a_c^2}$), which corresponds to 2.82 g (or approximately 3 times the Earth gravity). This SHG condition was continuously maintained from 0 to 6 days post fertilization, only stopping the centrifuge during 10 minutes daily to asses survival and hatching rate. At the end of the 6 day we observed and assessed the physiologic changes and behavioral traits.

2.4. RNA Isolation & Gene expression

Total RNA from N=10 larvae treated and N=10 larvae control were isolated using TRIzol (T9424, Sigma310 Aldrich, St. Louis, Missouri), according to the manufacturer's instructions, and quantified with ND-1000 spectrophotometer (NanoDrop Technologies). RNA (100 ng) was DNase I-treated (Thermo Fisher Scientific 315 Inc., Wilmington, DE, USA) to remove genomic DNA contamination and reverse transcribed into cDNA with SuperScript III RNase 316 Transcriptase (Invitrogen, Spain) with Random hexamer (Invitrogen, Spain), all according to the manufacturer's protocol.

Quantitative PCR (qPCR) was performed using 5 uL 2X qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems), cDNA was diluted 1:10 with DNase free water and 10 µL of the dilution was used as a template for qPCR, each reaction additionally contained 0.5 µL of each primer (Forward, and Reverse), and 2 µL of DNase free water. qPCR was carried out in technical replicates (3) for each sample.

2.5. Statistical Analysis

We assessed 4 biological replicates to study survival and hatching rates (n= 220 Control, n= 440 SHG). Data from survival were expressed as medium rate ± S.E.M. while data from hatching are represented as the logarithmic transformation of rate, and normality was evaluated with a *Kolmogorov–Smirnov test*, and Levene's test was used to assess homoscedasticity of variances.

3. Results

3.1. Survival and hatching

Survival was not affected by SHG treatment (Figure 2), however so far we observed a delay in the hatching time in the SHG larvae treated (P=0.004326), it was evident between the 2- 3 dpf (Figure 3).

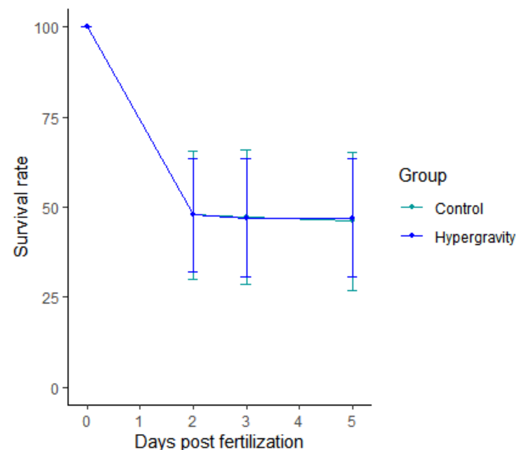

Figure 2. Survival rate of control and SHG larvae during 5 days of treatment. Data are shown as mean ± SE of 4 biological replicates. N=440 and N=220 individuals were used for both conditions, SHG, and control, respectively. No significant differences were found between the groups.

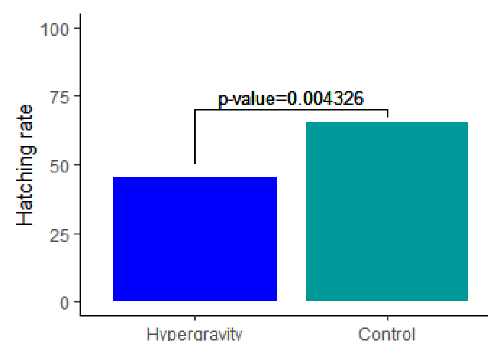

Figure 3. Hatching rate of larvae at 2 dpf treated with SHG compared with the control group. Four biological breeding pairs were used, with a total number of larvae of 208 and 273 in control and SHG, respectively. The bar graph represents the logarithmic transformation of hatching rate, finding significant difference (P=0.004) between control and SHG treated group. Normality was evaluated with a Kolmogorov–Smirnov test, and Levene's test was used to assess homoscedasticity of variances.

3.2. Physiology and Morphology

At the 6 day of exposition the larvae were observed and recorded to analyse the morphology and some behavioural features as

swimming, position, and movement frequency. And we found that in the SHG treated larvae was frequent and statistic significant abnormal features, such as vertical ascendent position with a 47% of the larvae in this position s ($P<0.05$), jerky movements in the 32% ($P=0.0003278$), and low movement frequency ($P<0.05$).

Regarding to the morphology we could observe that SHG treated larvae after 6 days present abnormalities in traits such as, body shape, flat tail and abnormal eyes size are illustrated in (Figure 4)

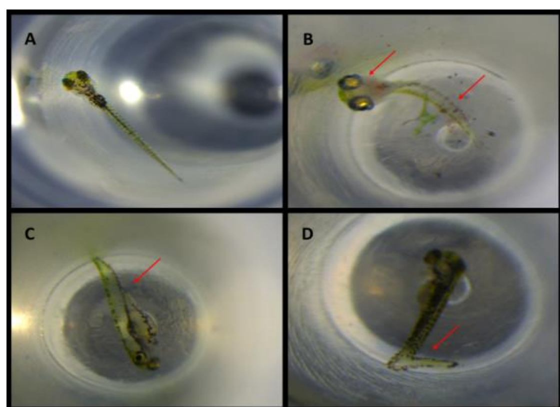

Figure 4. Morphology of the 5 dpf zebrafish larva. A: Dorsal view of a normal control zebrafish larva, B-D: Different views of SHG larvae exposed that presents alterations in its ocular, head, and tail morphology (arrows), also are in abnormal positions (B: Vertical ascendent position, C: Horizontal lateral position, D: Vertical descendent position).

3.3. Gene Expression Response

SHG exposure of zebrafish larvae caused the upregulation of two epigenetic markers, the *dnmt3 and tet1.* The expression on both of them showed significant differences regarding the control gene, the *dnmt3* in 4.4 fold change, and *tet1* in 3.2 folds with a *p* value< 0.01. On the other hand, the expression of *dnmt1* did not show a difference between treatments (Figure 5).

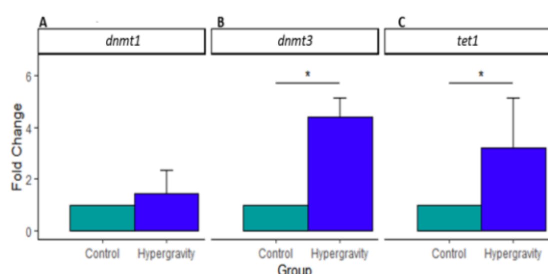

Figure 5. Gene expression of epigenetic markers. A: *dnmt1*, B: *dnmt3* and C: *tet1* expression profiles after 5 days of SHG exposure in N=10 larvae per group (control and SHG). Data are shown as mean ± SEM of fold change using control values set at 1. Significant differences (*P*<0.05) are symbolized by asterisks between treated and control groups. Gene specific primers for qPCR were mentioned in Table 3. The details of qPCR conditions were described in the materials and methods section.

4. Discussion

4.1. Survival and Hatching

According to our results survival is not affected by SHG, which is consistent with the findings by Wacker [10] whom neither found correlation between survival, and exposure to altered gravity. On the other hand, hatching rate showed a significant delay between 2 and 3 day post fertilization. Some studies have shown that changes in environmental conditions such as changes in temperature [11], oxygen level [12] could produce stress resulting in a hatching delay in zebrafish larvae. Our study is the first one to show the hatching effects of altered gravity is caused by mechanical stresses due to the container as a byproduct of the hypergravity.

4.2. Physiology and Morphology

Physiology and morphology was significantly affected by SHG. Those larvae exposed to 3 *g* had difficulties to acquire the normal position, and also showed changes in behavior as jerky movements and anormal swimming. In larval zebrafish, only the utricular otolith is responsible for gravity sensation [13], [14]. The vestibular system encodes information about head movement in space; both translational acceleration and tilt with respect to gravity are encoded by otoliths in the inner ear [15]. In unusual environments, such as in altered gravity, some of this information varies or is lost, then the perception of the correct direction can be affected [16].

A critical period in which the vestibular system is developed in zebrafish larvae begins before 30 hours post-fertilization (hpf) and ends after 66 hpf [13], then the exposition to altered gravity affects the normal growth and development of otoliths, which causes modification in the orientation patterns [17]. Modifications in normal position and orientation due to alterations in the vestibular system after gravity changes, also have been exhibited in murine [18], mollusks and amphibians [19], among others.

Morphological traits also showed alterations: the body shape, the disposition and shape of tail, and head size exhibited abnormal sizes and shapes. Other studies in zebrafish revealed that after exposure to altered gravity morphometric alteration have been presented in cranial bones [69], and in the musculoskeletal system [20].

4.3. Gene expression

An important finding of this study is that simulated hypergravity exposure can affect the normal expression of genes that are implicated in the regulation of some epigenetic mechanisms. Hence it may induce changes in DNA methylation status in the genome. While several studies in altered gravity have reported changes in gene expression, there are only a few that report how changes in gravity produce epigenetic alterations.

The high expression that showed *dnmt3* in our study agrees with the results obtained in rats that were exposed to 2 *g* hypergravity during 7 days in which upregulation of these genes was observed [21]. Studies carried out in human lymphocytes subjected to simulated microgravity during 72 h also obtained an up-regulation in *dnmt3* gene [22].

Our qPCR results showed a high level expression in *tet1*) in the SHG exposed larvae compared with the control group, but no studies in altered gravity have evaluated *tet1* as an epigenetic marker, however the role of this gene is well known. *Tet1* facilitates DNA demethylation of regulatory regions linked to genes involved in developmental processes [23]. *Tet* dysregulations in zebrafish have resulted in abnormal phenotypes and embryonic lethality [24]. This is an indication that *tet* proteins are dispensable for the correct organ development and body plan formation [24], [25], and thus changes in gene expression occurred by gravity can trigger alterations in phenotypical traits during early development.

5. Conclusions

Our study shows that gravity alteration has an impact not only in physiology but also in behavioral aspects in zebrafish larva. This study provides the first evidence for hatching responses induced by altered gravity conditions in fish. Moreover, hypergravity impacts the gene expression in genes implicated in epigenetic mechanisms. In all, we can conclude that zebrafish is a good *in vivo* model to study gravity effects to better understand epigenetic alterations along early development. The finding of this study also provides the basis for further research on the role of hypergravity-induced epigenetic changes in the regulation of gene expression and associated adverse environmental effects on zebrafish development.

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