# Epigenetic and physiological alterations in zebrafish subjected to hypergravity

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## Abstract

Gravity is one of the most constant environmental factors across Earth's evolution and all organisms are adapted to it. Consequently, spatial exploration has captured the interest in studying the biological changes that physiological alterations are caused by gravity. In the last two decades, epigenetics has explained how the environmental cues are able to altered gene functions in the organisms. Although many studies addressed gravity, the underlying biological and molecular mechanisms that are occurred in altered gravity for those epigenetics-related mechanisms, are mostly inexistent. The present study addressed the effects of hypergravity on development, behavior, gene expression, and most importantly, on the epigenetic changes in a world-wide animal model, the zebrafish (*Danio rerio*). To perform hypergravity experiments, a custom-centrifuge simulating the large diameter centrifuge (100 rpm ~ 3 *g*) were designed and zebrafish embryos were exposed during 5 days post fertilization (dpf). Results showed a significant decrease of survival at 2 dpf but not significance in the hatching rate. Physiological and morphological alterations including fish position, movement frequency and swimming behavior showed significant changes due to hypergravity. Epigenetic studies showed a significant hypermethylation of the genome of the zebrafish larvae subjected to 5 days of hypergravity. A downregulation of the gene expression of three epigenetic-related genes (*dnmt1*, *dnmt3*, and *tet1*), although not significant, were further observed. Taken altogether, gravity alterations affected biological responses including epigenetics in fish, providing a valuable roadmap of the putative hazards of living beyond Earth.

**Keywords**: space, epigenetic, gravity, genes, aquaculture, fish, methylation

## Introduction

Gravity is a fundamental physical component in the Earth's environment, and it plays a crucial role, shaping the evolution of all life forms (Volkmann & Baluska, 2007). The comprehension of the effects of this critical environmental factor is required for astronauts and future settlements beyond Earth. Thus, since the beginning of the spatial exploration, numerous studies in altered gravity have been performed in both microgravity and hypergravity conditions (reviewed in (Hariom et al., 2021). While microgravity experiments are complex as they require the presence of samples in the space —or in some cases by using microgravity simulator on Earth as clinostats with their technical limitations— (Herranz et al., 2013), hypergravity experiments can be easily simulated on Earth and, consequently, more convenient and considered as ground-based studies.

For many years, hypergravity has been used as a model for understanding the gravity alteration in the space. Hypergravity refers to the conditions where the gravity exceeds that on the Earth's surface (Wade, 2005). These hypergravity studies usually use a centrifuge machine with a large enough radius that shear forces in the sample (van Loon et al., 2004; van Loon, J.J.W.A. et al., 2005). Hypergravity has the capacity to induce significant alterations in various physiological systems within the body, and large literature is found related to physiological alterations. For instance, it has been observed that hypergravity can impact the immune systems of various species throughout evolution, ranging from humans and mammals (Boonyaratanakornkit et al., 2005; Rykova et al., 2008; Q. Li et al., 2013), to amphibians (Boxio et al., 2005; Frippiat, 2013), fish (Ohnishi et al., 1998), and insects (Marcu et al., 2011; Taylor et al., 2014). Vestibular system is one of the most studied systems due to its role in maintaining body equilibrium within Earth's gravitational field. This sensory apparatus which coordinates balance and movement is altered under gravitational variations resulting orientation problems to astronauts (Anken & Rahmann, 1999; Lawson et al., 2016).

Gravity alteration experiments have been conducted using a wide range of animal models, including *Drosophila* (Bae et al., 2016), *Caenorhabditis elegans* (Honda et al., 2014), mice and rats (Ross, 1994; Jamon, 2014), and fish (Aceto et al., 2016). Among these, zebrafish (*Danio rerio*), a tropical freshwater fish, belonging to the teleost family, has become an unprecedented tool for research in a variety of fields, such as genetics

(Norton & Bally-Cuif, 2010), development (Yalcin et al 2017), toxicology (Dai et al 2014), physiology (Briggs, 2002), aquaculture ( Ribas & Piferrer, 2014; Piferrer & Ribas, 2020), among others. Its short generation time, the large amount of fertilized eggs, its transparency during early development, the high similarity with human orthologous genes (~70%) and, the availability of genomic resources, make zebrafish an excellent animal research model. Besides the above-mentioned features, zebrafish have an additional advantage due to its physical nature, as it is born and bred in a neutral gravitationally environment, i.e., aquatic, which make an absence of body weight related to proprio-perceptions, reduce influence of gravity on supporting tissues and muscle and, higher sensitivity due to relative larger otoliths with differently positioned sacculus-otolith membranes (Rahmann & Anken, 2000). These characteristics make zebrafish an excellent model for microgravity and hypergravity studies and so a valuable tool in the current space exploration era. In fact, a variety of studies has been evaluated the effects of gravity in zebrafish. One of the most studied system in zebrafish is the vestibular system (Moorman et al., 2002; X. Li et al., 2011, 2017), where it has been found that microgravity affects the otolith development. Alterations in genes involved in lens development were described (Shimada & Moorman, 2006), together with hematopoiesis- and cardiovascular-related genes showing that short-term hypergravity induced physiological changes in the zebrafish embryos (Hariom & Nelson, 2022).

Epigenetic mechanisms are responsible for the alteration of the final phenotype under environmental pressure, and it is defined as permanent changes in gene expression that occur without modifying the nucleotide sequence of the genome (Qiu, 2006; Deans & Maggert, 2015). One of the most studied epigenetic mechanisms is DNA methylation which plays a key role in regulating cellular processes in living organisms (Laird, 2010). This type of modifications implies the addition of a methyl group to the 5' position of cytosine (5mC) cytosine-phosphateguanine (CpG) dinucleotides, named as CpG sites (Bird, 1986). DNA methylation is performed by enzymes known as DNA methyltransferases (dnmts) that catalyze the methylation reactions (Miranda & Jones, 2007; Esteller, 2008). In mammals, the main dnmts include DNA methylation transferase 1 (dnmt1) responsible to maintain DNA methylation levels in the cells, and another two, dnmt3a and dnmt3b, for *de novo* DNA methylation (Pradhan et al., 1999; Okano et al., 1999). To maintain the genomic methylation homeostasis, cells rely on

these DNA methyltransferases but also to demethylase enzymes. DNA demethylation is a complex process, not fully understood, in which 5mC are converted to 5-hydroxymethylcytosine (5hmC) by the ten-eleven translocation (Tet) family of dioxygenases (Tahiliani et al., 2009). Among different tet members, tet1 is the most studied because plays a key role in the demethylation process preventing DNA from methylation maintenance (Zhao & Chen, 2013).

For the epigenetic mechanism being stable, three basic components are required: epigenator, epigenetic initiator and epigenetic maintainer (Piferrer, 2018; Balasubramanian et al., 2019). An epigenator is a crucial signal responsible for initiating the intracellular pathway in response to environmental stimuli. It can be any factor or event that triggers the activity of the initiator molecule (Berger et al., 2009). Remarkably, any environmental change, regardless of its nature, has the potential to act as an epigenator and producing a signal that persists long enough to exert a significant impact on the epigenetic phenotype (Herceg & Vaissière, 2011; Schübeler, 2015). Thus, gravity alteration is an epigenator. Although epigenetics has been extensively studied in various research areas over the last two decades, research specifically involving epigenetic modifications in altered gravity conditions remains limited. In *Arabidopsis in vitro* cultures, both microgravity and hypergravity experiments resulted in increased DNA methylation (Kamal et al., 2018). Transcriptomic studies in rats revealed that genes related to the DNA methylation machinery were altered (Casey et al., 2015). Similarly, in cultured human cells, microgravity altered the methylome together with transcriptome harboring the understanding of molecular events (Chowdhury et al., 2016). In 2019, a National Aeronautics and Space Administration (NASA) experiment on twins deciphered multidimensional data after a one-year-long human spaceflight, revealed DNA methylation changes in immune and oxidative stress-related pathways, along with other alterations at various cellular levels (e.g., microbiota, metabolism, transcriptome, or body mass) (Garrett-Bakelman et al., 2019). Although current data proposed that the adaptation to gravity alterations may proceed through epigenetic changes, more research in this flourishing field needs to be explored before the space environment being a safe place to be. To date, no altered-gravity studies involving zebrafish and epigenetics have been reported yet. Thus, in order to tackle space-incurred epigenetic disturbances, here it is examined, for the first time, the epigenetics changes by analyzing the global DNA methylation patterns and the gene expression of three

164 epigenetic-related genes (*dnmt1*, *dnmt3* and *tet1*). To shed light on the side effect of
165 hypergravity during zebrafish development, fish survival, hatching rate and
166 physiological traits, were also addressed.
167
168 **Materials and methods**
169
170 ***Zebrafish husbandry***
171 Zebrafish (TUE strain) were housed in the animal facilities of the experimental
172 aquariums zone (ZAE) at the Institute of Marine Sciences (ICM-CSIC, Barcelona,
173 Spain). Fish were held in 9 liters (L) tanks on a recirculating system (Aquaneering, San
174 Diego, CA) in a chamber with a photoperiod of 12 h of light and 12 h of darkness, an air
175 temperature of 26 ± 1 ◦C and a humidity of 60 ± 3%. Physicochemical parameters were
176 monitored daily, staying at appropriate conditions ( Ribas & Piferrer, 2014; Piferrer &
177 Ribas, 2020): water was maintained at (28 ± 0.2°C), pH (7.2 ± 0.5), conductivity (750–
178 900 µS) and dissolved oxygen (6.5–7.0 mg/l) with a water pump of 3,000 L/h and a UV
179 light system to eliminate any possible bacteria in the water. Sulfite, sulfate, nitrate and
180 ammonia quality parameters were checked weekly using commercial kits. Adult fish
181 were fed twice daily, receiving dried food and live *Artemia nauplii* (AF48, INVE
182 Aquaculture, Dendermonde, Belgium).
183
184 ***Hypergravity device***
185 A custom-made hypergravity centrifuge was performed by using a regular laboratory
186 rotary mixer with a maximum speed of 100 rpm (model: ANR100DE, OVAN
187 laboratory equipment) by adding two perpendicular arms of R=25 cm ending in two
188 opposite gondolas to support an experimental plate (Figure 1A, B). This setup allowed
189 achieving a gravity-like acceleration in up to ~3 $g$ (where $g$ refers to 9.81 m/s$^2$). To
190 maintain the required environmental conditions for the larvae (27-28°C and 60-65%
191 humidity) during the hypergravity experiments a methacrylate box was adapted with a
192 heating system using ProClima v8.1.6.1 software (Schneider Electric 2020). These
193 parameters were checked daily. The hypergravity device was set up to 100 rpm
194 (revolutions per minute, or 10.47 rad/s) spin delivering a centripetal acceleration ($a_c$) of
195 27.4 m/s$^2$ that vectorially added to the existing Earth acceleration ($g$) of 9.81 m/s$^2$,
196 resulting in total acceleration $a_T$ = 29.1 m/s$^2$, which corresponds to 2.96 $g$ (or

197 approximately 3 *g*). The geometry and the justification for these calculations are
198 illustrated in Figure 1C.
199
200 ***Experimental design***
201 Zebrafish pairs (one female and one male) were bred by transferring to breeding tanks
202 with a transparent separator overnight. The following day, the separator was removed
203 and fertilized eggs were collected and maintained in Embryo Medium Solution (EMS,
204 Ph= 7,2; NaCl 0.8 g/L, KCl 0.04 g/L, $NaH_2PO_4$ 0.0036 g/L, $H_2KO_4P$ 0.006 g/L, $CaCl_2$
205 0.144 g/L, $MgSO_4$ 0.12 g/L, $NaHCO_3$ 0.35 g/L, and 20 µl/L Trypan blue as antifungal).
206 The total number of fertilized eggs was counted to guarantee fertility according to the
207 reference values for this species and fish post-hatch survival agreed with OECD's
208 guidelines for the Fish Sexual Development Test (OECD, 2011).
209
210 Fertilized eggs were collected and placed individually into 96 wells plates with 250 µl
211 of EMS and were covered with adhesive sealer to avoid fluid loss. For each biological
212 pair, embryos were distributed in three different plates: control group placed inside the
213 methacrylate box, hypergravity group placed into the gondolas, and mock group (MG)
214 placed in the zebrafish embryonic incubator (28°C) outside the methacrylate box. The
215 MG was used as an internal control of the standardized embryonic development in this
216 fish species (Kimmel et al., 1995). The experiment was conducted six times, with each
217 replication using different single pairs to ensure biological replication. In total, there
218 were 440 individuals analyzed, with 220 individuals in the control group and 220 in the
219 hypergravity group.
220
221 To evaluate the survival and hatching rate, the hypergravity device was stopped during
222 6-10 minutes every 6 hours until 100% of the larvae were hatched (i.e., 3 dpf). After
223 that, the device was stopped every 24h. Embryonic and larvae development for each of
224 the six groups three groups were observed by a Leica EZ4 Stereo Microscope (Leica
225 Microsystem Ltd.). After maximum of 10 minutes, the embryos were placed on their
226 respective experimental conditions until the end of the experiment.
227
228 ***Ethogram activity***
229 At 5 days of experiment, the larvae were observed and recorded to assess their ethogram
230 activity. This analysis consisted on identifying three locomotor aspects: position,

movement frequency and swimming behavior. Table 1 described each of the observed characteristics for each larva. Larvae were observed and recorded individually for each of the experimental groups with a total of 180 observations (N= 6 biological groups, N= 15 larvae hypergravity, N= 15 larvae control group). Teratologies resulting from the treatment were carefully observed and recorded using the Stereo Microscope (Leica Microsystem Ltd.). To minimize inter-observer error bias, all analyses were performed by the same researcher.

***DNA extraction***

A total of 10 larvae for each control and hypergravity groups (N=20) for one biological replicate were digested overnight at 56°C with buffer containing 1 µg of proteinase K (Sigma-Aldrich, St. Louis, Missouri) to eliminate proteins. Then, the standard phenol-chloroform-isoamyl alcohol protocol (PCI 25:24:1) with 0.5 µg ribonuclease A (PureLink RNase A, Life Technologies, Carlsbad, California) was performed to isolate DNA and

eliminate RNAs. The quality and quantity of DNA was measured by Qubit (Thermo Fisher Scientific, Waltham, Massachusetts). Isolated DNA samples were stored at −20°C until further analysis.

***Global DNA methylation analysis***

Global DNA methylation was performed in genomic DNA using 5-mC DNA ELISA kit (Zymo Research, USA) following manufacturer's protocol and that described in (Valdivieso et al., 2020). Briefly, 100 nanograms (ng) of each DNA sample was used for analysis. The standard curve was prepared by mixing negative and positive controls at different proportions. The final methylation concentrations of standards were 0%, 5%, 10% 25%, 50%, 75%, and 100%, respectively. The absorbance was measured at 405 nm using an ELISA plate reader (Infinite® 200 PRO, Tecan™). All samples were analyzed in duplicates. The percentage of 5-mC for unknown DNA samples was calculated using the equation: $\% 5-mC = e\{(Absorbance - y - intercept)/Slope\}$ . Percent 5m-C values were corrected with the zebrafish CpG density according to the manufacturer instructions. The percentage of CpG was calculated according with the formula presented by Valdivieso et al., 2020 (Valdivieso et al., 2020), where the latest zebrafish genome from Ensembl was downloaded (www.ensembl.org). Then, the length of the genome (L) = 1,674,207,132 bp was extracted, and the total number of

cytosines (C) and the number of CpG dinucleotides (CG) were calculated. Once the total number of C = 306,412,859 and CG = 29,220,867 were obtained from the zebrafish genome, the fold difference of CpG density (total CG genome/L) between the genomes of (*E. coli/D. rerio*) = (0.07472/0.0175) = 4.2811 was calculated. Finally, to obtain the global methylation values, the % 5m-C/CpG density values were multiplied by the value obtained from the total number of C/L zebrafish = 0.1830.

***Gene expression analysis***

RNA was individually extracted from 10 larvae each group (control and hypergravity) for one biological replicate (N = 20 total) with TRIzol (T9424, Sigma-Aldrich, St. Louis, Missouri) according to manufactured procedures. RNA pellets were suspended in 25 µl DEPC–water and kept at -80°C. RNA concentration was determined by ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality was checked on a 1% agarose/formaldehyde gel. Following supplier protocols, 100 ng of total RNA for each sample were treated with DNAse I, Amplification Grade (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and retrotranscribed to cDNA with SuperScript III RNase Transcriptase (Invitrogen, Spain) with Random hexamer (Invitrogen, Spain).

Quantitative Polymerase Chain Reaction (qPCR) was performed using synthetized cDNA, previously diluted 1:10 with DNase free water, 5 µl of 2X qPCRBIO SYBR Green Mix Lo-ROX (PCR Biosystems), 0.5 µL of each forward and reverse primers, and 2 µL of DNase free water. qPCR was carried out in technical triplicates for each sample. The conditions in the thermocycler were as follows: initial denaturation for 3 min at 95°C, 39 cycles of 10 s at 95°C, 30 s at annealing temperature; followed by melt curve analysis (65°C–95°C at 0.5°C/5 s) to verify amplification of a single product. Dissociation step, primers efficiency curves and PCR product sequencing confirmed the specificity for each primer pair. All primers efficiencies ranged between (95-104%). Primer sequences were designed using Primer3web v4.1.0 (Koressaar & Remm, 2007) and further information is found in Table 2.

***Statistical analysis***

All statistical analyses were conducted using R (v. 1.1.456). The *homogeneity of variances* was assessed using *Levene's* test, followed by a *Shapiro-Wilk* test to check for

normality of the data. For normally distributed data, we performed an *ANOVA*, and for non-normal data, we utilized non-parametric tests such as *Kruskal-Wallis*.

To assess differences between groups and categories in the ethology, we employed the Chi-square test. Statistical significance for all evaluated parameters was considered at $P<0.05$. Graphs were created using the *ggplot2* package (v.3.1.0) by Wickham (2016)

## Ethics statement

The procedure for using zebrafish in this study was conducted following approved guidelines by the Bioethical Committee of the Generalitat de Catalunya (reference code 9977) and the Spanish National Research Council (CSIC) Ethics Committee (reference code 1166/2021). In the present study, the fish rearing and maintenance were following the European regulations of animal welfare (ETS N8 123, 01/01/91 and 2010/63/EU). Fish facilities in the ICM were validated for animal experimentation by the Ministry of Agriculture and Fisheries (REGA number ES080190036532).

## Results

### *Survival and hatching*

The survival of zebrafish embryos and larvae exposed to hypergravity was determined every dpf during the experiment. As shown in Figure 2, the survival was significantly affected at 2 dpf decreasing embryonic survival. In contrast, no differences were found during larva development until the end of experiment between treatment groups. Hatching rate was not significantly affected by the hypergravity condition, occurring between the second and third day both in control and in hypergravity, as it normally occurs in zebrafish (Figure 3). Most of the hatching occurred at 58 hpf in both groups and at 72 hpf the hatched was completed for all the experimental individuals in both groups. However, at 52 and 64 hours post fertilization (hpf), the hatching rate was decreased in hypergravity when compared to control, from 15 to 10% and 90 to 80% in control and hypergravity, respectively.

### *Ethogram analysis*

The locomotor activities of exposed larvae were evaluated after 5 days of hypergravity. The observation of the fish position, movement frequency and swimming behavior showed high significance in almost all the studied characteristics (Figure 4 and 5). For control, 75.40% of the larvae were found in horizontal (normal position) whereas only

18.26% of the larvae in hypergravity showed this same position (Figure 4A). The most observed position for hypergravity exposed larvae was the vertical ascendant position with a 47.10% while in control only a 11.50% of the individuals presented this position. Vertical descendent position was present in 13.50% of larvae subjected to hypergravity while none of the control was observed.

More than 90% of the larvae in the hypergravity had no movement frequency (59.6%) or low movement frequency (30.8%) and only 3.8% and 5.8% had high or medium movement frequency. In contrast, ~51% of the larvae in control showed high or medium movement frequency and the other half presented low (16.4%) or static (32.80%) movement frequency (Figure 4B).

Hypergravity was able to altered significantly the swimming behavior by increasing jerky movements (32.70%) and wrong swimming (7.70%) and, decreasing larvae with normal swimming when compared to control conditions (Figure 4C). No significance was found for Erratic swimming individuals between groups.

Some zebrafish larvae showed teratologies after the hypergravity treatment. These teratologies consisted four major types based on their position: body curvature, tail curvature, abnormal eyes size, and overall body deformation (Figure 5).

***Methylation alterations***

Hypergravity exposure was able to alter significantly the global DNA methylation levels between the control and the hypergravity group (*t*-test, $P < 0.007$, Figure 6). Larvae in hypergravity presented hypermethylation levels of the global DNA when compared with the control group (percent CpG mean was 21.04% and 26.53%, in control and hypergravity groups, respectively).

***Gene expression response***

Hypergravity exposure of the zebrafish larvae caused downregulation of the three studied epigenetic markers. The fold change was -1.6, -1.5, and -1.6 for *dnmt1*, *dnmt3b* and *tet1*, respectively, although without significance (Figure 7).

## Discussion

366 ***Hypergravity influenced larvae survival but not altered hatching rate***

367 Very limited information exists on the impact of altered gravity on fish survival and
368 hatching success. In the present study, hypergravity was able to decrease embryonic
369 survival at 2 dpf just before larvae started to hatch. In contrast, hatching rate was not
370 significantly affected by hypergravity, although slight decreased the hatching rate at 52
371 and 68 hpf. Almost 40 years ago, the first fish on the space base station Skylab 3, mud
372 minnow (*Fundulus heteroclitus*) eggs were able to hatched successfully with a rate of
373 96% indicating that gravity alteration did not affect the hatching rate (Scheld et al.,
374 1976; Johnson, 2016). Gravity alteration did not affect medaka (*Oryzias latipes*) as
375 embryos hatched after being fertilized on space but also those space-fertilized eggs after
376 being sent back to Earth after 3 days of landing (Ijiri, 1994; Ijiri, 1995; Ijiri, 1998).
377 Similarly, analogous space-related experiments in European sea bass (*Dicentrarchus*
378 *labrax*) and meagre (*Argyrosomus regius*) showed no significant difference after
379 exposing eggs in a simulating spacecraft launcher vibration (Przybyla et al., 2020).
380 Thus, overall current available data indicate that hatching might be not sensible to the
381 environmental stress caused by gravity alterations, probably due to the natural
382 protection of the chorion to external stimulus.

383

384 ***Hypergravity affected the morphology and behavior***

385 In the zebrafish model, larvae exhibit mature swimming between 4-5 dpf and respond to
386 visual and stress stimulus increasing the movement (Basnet et al., 2019). Zebrafish are
387 sensitive at a range of external stimuli such as olfactive, sensitive, vestibular inputs,
388 heat and vision and many studies in zebrafish showed that swimming is altered under
389 gravity modification (Anken & Rahmann, 1999; Ibsch et al., 2000; Colantonio et al.,
390 2009). In the present study, after hypergravity exposure, fish position, movement
391 frequency and swimming behavior showed drastic significant changes. Swimming
392 behavior was affected by the increase of the gravity and larvae showed atypical
393 displacements such as jerky movements and wrong swimming. Most of the tested
394 individuals subjected to hypergravity-treated were positioned in vertical ascendant and a
395 static or with low movement, indicating the environmental stress impact by the
396 hypergravity environment. On space, mummichog fish swam in elongated loops, named
397 as looping response, during the first three days, as they were disoriented because the
398 vestibular system was affected (Von Baumgarten et al., 1975). Looping appeared to be
399 the fish's equivalent of space sickness, although it gradually disappeared as the fish

learned how to orient themselves (R. W. Hilbig & Anken, 2017). Thereafter, fish living on space swam in regular patterns, oriented by artificial light, and even were able to mate naturally (Ijiri, 2003).

Thigmotaxis is a valid index of anxiety in which animal prefers to be closed to the walls in vertical position and its evolutionarily conserved across different species, such as fish, rodents, and humans (Lamprea et al., 2008; X. Liu et al., 2016; Basnet et al., 2019). In fact, vestibular system is responsible of the fish orientation and it is directly affected by gravitational changes (Rahmann & Slenzka, 1994; Rahmann & Anken, 2000). A complete map of the gravity-sensing system straddling from the inner ear to the brainstem was described (Z. Liu et al., 2021). In fish, otoliths, the inner ear heavy stones, are responsible for fish orientation and their growth were targeted in space-related research (Kondrachuk & Boyle, 2006; X. Li et al., 2011). Hypergravity experiments in zebrafish and Cichlid fish embryos (*Oreochromis mossambicus*) showed an increase of the otolith's growth (Anken et al., 1998, 2001, 2002; Wiederhold et al., 2003) while microgravity on space flight or by clinostats yielded an opposite growing effect in swordtail (*Xiphophorus helleri*), in cichlid fish larvae (Anken et al., 2010; Wiederhold et al., 2000) and in zebrafish embryos (X. Li et al., 2011). Generation of a mutant medaka strain ha (genotype ha/ha) with an absence of otoliths, decreased the sensitivity to gravity after microgravity and parabolic flights (Ijiri et al., 2003).

Three weeks of hypergravity experiment with cichlid fish (*Oreochromis mossambicus*) larvae, the morphogenetic development and the swimming was not affected. However, when the centrifuge treatment stopped, looping and spinning movements appeared (Rahmann et al., 1996). However, within hours, this kinototic behavior disappeared. Similar results were observed in the present study because after some days of hypergravity treatment the abnormal movement disappeared and fish developed normally (Salazar-Moscoso, personal comments). Similarly, medaka fish had looping response during three days after landing on Earth, after that, they swam properly (Ijiri, 2003). Overall results may indicate that zebrafish is able to adapt to the novel environments thanks of vestibular neuronal system plasticity (Straka et al., 2005; Almeida & Lyons, 2017).

Some teratologies were observed due to hypergravity, such as body curvature or body deformation. The data presented here aligns with previous findings in zebrafish subjected to similar hypergravity conditions (3 g, from 0 to 5 dpf). These zebrafish exhibited morphometric changes, including an enlarged head and increased cranial bone formation. These findings supported that hypergravity can induce consistent effects on the developmental processes in zebrafish (Aceto et al., 2015).

***Genome-wide DNA methylation levels were altered***

One of the most important finding of this study was that hypergravity exposure of zebrafish during 5 days, regulated the epigenetic events by hypermethylating genome-wide DNA levels. Few studies addressed the epigenetic changes in the genome due to gravity alterations, in particular to hypergravity. To date, and based on our knowledge, this is the first time reported in fish.

DNA methylation levels were altered in response to long-term isolation experiments, for example in mice in the SpaceX-4 mission increasing the total genome methylation (Ogneva et al., 2018). DNA methylation dynamics were analyzed in a long-term isolation of simulated space travel in the blood of the crew of the Mars-500 mission, identifying six significant epigenomic patterns at post-isolation recovery (Hou et al., 2022). The functions of these DNA methylation patterns were mostly related with the immune-system and tumors but also epigenetic genes related to glucose and mood-state disturbance were observed (Liang et al., 2019). As expected, by analyzing epimarkers for aging, the simulated space travel was associated with significant decreases in epigenetic aging meaning that the stressed produced by the travel decreased the biological age of the crew (Nwanaji-Enwerem et al., 2020). In mice, epigenetic memory was analyzed by the retinal profiles of animals exposed to microgravity and irradiation four months after the exposition (Kothiyal et al., 2022). Their results revealed a crosstalk between the epigenome and the transcriptome as 23 potential biomarkers genes related to retinal function and inflammatory response showed significant changes (Kothiyal et al., 2022). In this sense, in cultured human lymphoblastoid cells revealed that microgravity induced a ~60% hypomethylation and ~92% hyperhydroxymethylated regions of the methylome together with 370 transcripts associated with crucial biological processes (Chowdhury et al., 2016)

In fish, it is known that the environment-epigenetic interactions are responsible to determine the phenotype in those animals growing in artificial and cultured environments (Skinner et al., 2010; Feil & Fraga, 2012). In the last decade, many studies in fish recalled on the importance of epigenetics, both during early development and adulthood in cultured conditions. For example, high temperature —the most studied environmental factor in fish—, suffered during larvae stages in the hatcheries, was responsible to masculinize populations through epigenetic events (Ospina-Álvarez & Piferrer, 2008; Ribas, Liew, et al., 2017). These epigenetic events occurred by high temperature were inherited to the following generation (F1) (Valdivieso et al., 2020) evidencing the importance of epigenetics, not only for the current generation but for the futures. Another environmental factors, the density —the number of fishes in a tank—, caused masculinization in zebrafish (Ribas, Valdivieso, et al., 2017) revealing some epigenetic alterations in the fish gonads (Caballero, unpublished results). Additionally, environmental-epigenetics interactions after immune stimulation during sex differentiation in zebrafish showed and alteration of the methylation of some immune-related genes (Caballero-Huertas et al., 2020; Moraleda-Prados et al., 2021). The present data indicated that the epigenetic machinery was reprogrammed by gravity alterations, and although many data needs to be performed, in particularly whether zebrafish would be used as a space-related animal model, the underlying epigenetic alterations need to be considered to fully comprehend the gravity-epigenome interactions. In this sense, higher genome coverage techniques, such as whole-genome bisulfite sequencing (WGBS) or by a single-nucleotide resolution Nanopore sequencer, would bring better comprehension of the epigenetic dynamics under gravity alterations.

***Expressions of dnmt1, dnmt3 and tet1 downregulated without significance***

Epigenetics are dynamic through the life of an organism and are implicated in many developmental process, cell differentiation, genomic imprinting, and modulating gene expression (Hermann et al., 2004; Smith et al., 2011). Epigenetic mechanisms are responsible for permanent heritable alterations in the cellular gene expression. To gain insight into the molecular events underlying hypergravity's effects, we performed an analysis of three genes related to epigenetic regulation. After 5 days of treatment, there was an inhibition of *dnmt1*, *dnmt3* and *tet1* expression although no significant. The inverse correlation between the hypermethylation of the DNA levels found in the same experiment with the lower expression of these epigenetic-related genes, were in

accordance with the classical dogma; higher DNA methylation is associated with the inhibition of the gene transcription machinery (Jones & Takai, 2001; Deaton & Bird, 2011). Nevertheless, it is currently accepted that DNA methylation are dynamic and complex processes in which many genomic elements contribute to transcriptional regulation: exons (Brenet et al., 2011), gene body (Blattler et al., 2014), introns (Anastasiadi et al., 2018) and, post transcription modifications (Shilatifard, 2006).

Quite extensive bibliography refers to gene regulation in space exploration related studies. In a recent review, almost 200 articles were included and the alteration of genes in many organisms and biological system were described (Corydon et al., 2023). Thus, reflecting the importance of the knowledge of the consequence of gene expression to tackle the risk health for the astronauts and alive organisms. In zebrafish larvae, the gene expression in hypergravity (24 h, 3g) revealed differential expression of genes involved in the development and function of the skeletal, muscular, nervous, endocrine and cardiovascular systems (Aceto et al., 2015). Simulated microgravity affected the expression of some genes related to fish musculoskeletal, cardiovascular, and nuclear receptor systems (Aceto et al., 2016) and the immune system in response to a viral response (Zhu et al., 2021). In contrast, the number of articles addressing the expression of epigenetic related genes are much less extent, not only in fish, but also in other animals and humans, and some time contradictive. Likewise to the data obtained in this study, did not change the expression of TET1 and TET3 in cardiac and lung of mice 37 days onboard of the American International Space Station (ISS) of SpaceX-4 mission (Ogneva et al., 2018). DNMT1, DNMT3a, and DNMT3b decreased at 7 days in human T-lymphocytes microgravity exposed (Singh et al., 2010) and a transcriptomic study of pregnant rats subjected to spaceflight showed that DNMT1 was downregulated while DNMT3a was upregulated (Casey et al., 2015). In addition, mutation in the blood samples of space shuttle astronauts were identified in the DNMT3A and TP53 genes (Brojakowska et al., 2022). Overall data indicate that epigenetic-related genes are altered due to gravitational changes, but more research needs to be performed to fully understand the crosstalk between the epigenome and the transcriptome.

## Conclusions

This study presents the first evidence of epigenetic impacts on the DNA methylation levels in zebrafish subjected to hypergravity during early development. Although not

535 statistically significant, there was a noticeable downregulation tendency observed in
536 three epigenetic-related genes' expression. Furthermore, the survival rate decreased two
537 days after the treatment, while the hatching rate remained unaffected by hypergravity. In
538 contrast, physiological traits (position, movement frequency and, swimming behavior)
539 of the larvae were drastically affected, accompanied by the observation of some
540 teratologies.
541 The presented data and experiments explore the new domain of how altered gravity
542 impacts development in living models by, for the first time, looking into epigenetic
543 effects in fish. Future experiments in space (this time under microgravity but following
544 similar protocols) shall shed some light on whether development of adult and fertile
545 animals (and eventually humans) could develop in space or other planetary bodies.
546

547 **Acknowledgments**

548 This study was supported by the Spanish Ministry of Science and Innovation grant
549 2PID2020-113781RB-I00 “MicroMet” and by the Consejo Superior de Investigaciones
550 Científicas (CSIC) grant 02030E004 “Interomics” to LR. GA-E's work is supported by a
551 Ramón y Cajal 2018 fellowship RYC-2017-22489 from Agencia Estatal de
552 Investigación (AEI), resources from Unidad de Excelencia María de Maeztu CEX2020-
553 001058-M and the Generalitat de Catalunya/CERCA programme. In addition, this study
554 was supported by the Spanish government through the ‘Severo Ochoa Centre of
555 Excellence’ accreditation (CEX2019-000928-S) and JAEICU2021-ICE-CSIC to MS.
556 We thank the lab technician Gemma Fusté for her essential assistance in fish facilities.
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572 **Table 1**. Ethogram description of zebrafish observed in the experiments

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| Activity | Characteristic | Description |
|---|---|---|
| Position | Horizontal | Normal dorsal position |
| | Horizontal lateral | Lateral up position |
| | Vertical ascendant | Fish's body in vertical position and head up |
| | Vertical descendent | Fish's body in vertical position and tail up |
| Movement frequency | High | 5 or more movements in 30 seconds |
| | Medium | 2 to 4 movements in 30 seconds |
| | Low | 1 movement in 30 seconds |
| | Static | No movement |
| Swimming behavior | Normal swimming | Normal Swimming |
| | Erratic flotation | Swimming in circles, up and down |
| | Jerky movements | Fast and repetitive movements without displacement |
| | Wrong swimming | Swimming upside down or on their side |

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579 **Table 2**. Gene specific primers used for quantitative PCR

| Gene name | Abbreviation | Primer sequence (5'- 3') | Acc. No (GenBank) |
|---|---|---|---|
| Elongation factor 1 alpha | *ef1α* | F: CTGGAGGCCAGCTCAAACAT | NM_131263 |
| | | R: ATCAAGAAGAGTAGTACCGCTAGCATTAC | |
| Ribosomal Protein L13a | *rpl13a* | F: TCTGGAGGACTGTAAGAGGTATGC | NM_212784 |
| | | R: AGACGCACAATCTTGAGAGCAG | |
| DNA (cytosine-5-)-methyltransferase 1 | *dnmt1* | F: TCTTCAGCACTACAGTTACCAATCCT | NM_131189 |
| | | R:CGTGCACATTCCCTGACACT | |
| DNA (cytosine-5-)-methyltransferase 3 beta | *dnmt3b* | F: AAGATTTAGGCGTCGGTTTCG | NM_131386 |
| | | R: GTGTCACCCCCTTCAATTAACTG | |
| Ten-eleven-translocation 1 | *tet1* | F: TGACTCACCAGCACTTGAAAAC | KC689999.1 |
| | | R: TTGGTGTCCACATCAGCAGT | |

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584 **Figures**

585 **Figure 1. A)** A custom-made hypergravity centrifuge used to perform the experiments.
586 **B)** Gondola with the 96 well plate where the larvae were placed during the 5 days of
587 treatment. **C)** Graphical representation of the centrifuge and gondola showing the
588 variables and vectors used to calculate the value of the acceleration (artificial
589 hypergravity) at the center of the gondola. The centrifuge radius, R, is defined as the
590 distance of the center of rotation to the outer edge of the platter. The gondola arm length
591 l was measured from the outer edge of the platter of centrifuge to the end of the
592 gondola. Both of them (R and l) were measured with a tape measure. The angle, $\boldsymbol{\theta}$, was
593 calculated using taking an image and analyzing it using a program called *angulus* (DPP
594 v1 2020). r is the gondola radius, $v$ is the tangential velocity due to rotation, $ac$ is the
595 centripetal acceleration, and $\omega$ is the angular velocity.
596
597 **Figure 2.** Survival rates of control and hypergravity zebrafish larvae during 5 days of
598 treatment. Each data point shows the mean ± SE of six independent groups with a total
599 number of 220 individuals per condition (control and hypergravity). The cumulative
600 survival with different letters indicates a significant difference ($P < 0.005$) according to
601 the Least significant difference (LSD) test.
602
603 **Figure 3.** Hatching rate of larvae at 2 and 3 days post fertilization treated with
604 hypergravity compared with the control group. Six biological replicates were made,
605 with a total number of larvae of 220 and 220 in control and hypergravity, respectively.
606 Data are presented as percentage ± standard error of the mean (SEM). Normality was
607 evaluated with a Kolmogorov–Smirnov test, and Levene's test was used to assess
608 homoscedasticity of variances. No differences between groups were found according to
609 Least significant difference (LSD) test.
610
611 **Figure 4.** Ethogram analysis consisted on identifying three locomotor aspects: position
612 **(A)**, movement frequency **(B)** and swimming behavior **(C)** of larvae of zebrafish at 5
613 days post fertilization (dpf). Bar graphs representing the percentage of individuals in
614 control and hypergravity conditions. Six biological replicates were observed with a total
615 number of 90 individuals in the hypergravity group and 90 in the control. The different
616 colors represent the assessed features in each parameter. The data are presented as

617 percentages of individuals. To evaluate significant differences, we performed a Chi-
618 square test.* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.
619
620 **Figure 5.** Teratology observed at 5 days post fertilization (dpf) in zebrafish larvae
621 exposed to ~3 *g* hypergravity from 0 to 5 dpf. Positions of the larvae are showed in the
622 figure: horizontal (A), vertical ascendant (B); horizontal lateral (C); vertical descendent
623 (D). Teratologies included four major types: body curvature (B), abnormal eyes size
624 (B), overall body deformation (C) and tail curvature (D).
625
626 **Figure 6.** Global DNA methylation in zebrafish larvae after 5 days of ~3 *g*
627 hypergravity. N=10 larvae per group (control and hypergravity). Means were compared
628 with the Student's t-test. In the boxplot the thick line represents the median and the
629 datapoint outside corresponds to atypical values.
630
631 **Figure 7.** Expression of three epigenetic-related genes in zebrafish larvae after 5 days of
632 ~3 *g* hypergravity. Data are shown as mean ± SEM of fold change using control values
633 set at 1. N=10 larvae per group (control and hypergravity). No significant differences
634 were found.
635

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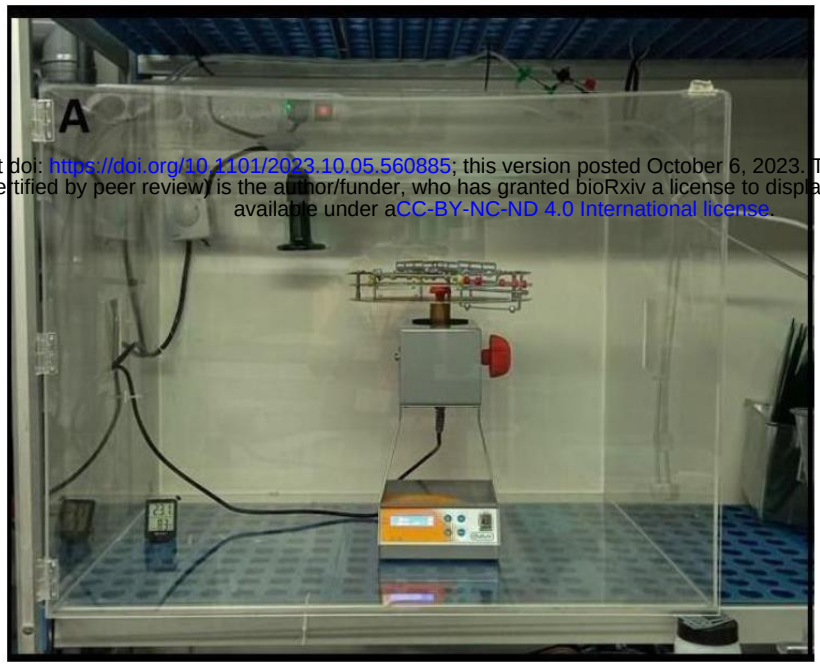
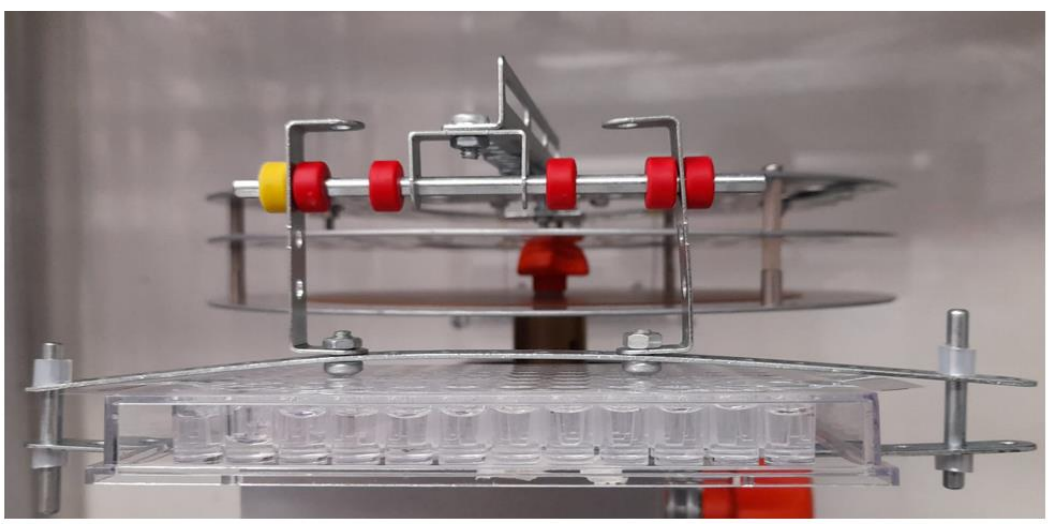
**A**




**B**



**C**