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From the cover: Montage of swimming *Danio rerio* (zebrafish) larvae. See article on page 37 of this issue. Photo by Pedro Llanos.
GENERAL INFORMATION

*Gravitational and Space Research* (ISSN 2332-7774) is a journal devoted to research in gravitational and space sciences. It is published by the American Society for Gravitational and Space Research, a non-profit organization whose members share a common goal of furthering the understanding of the effects of gravity and the use of the unique environment of spaceflight for research. *Gravitational and Space Research* is overseen by a steering committee consisting of the Publications Committee, the Editor, the President, and the Secretary-Treasurer of the ASGSR.

The American Society for Gravitational and Space Biology was created in 1984 and became The American Society for Gravitational and Space Research in 2012 to provide an avenue for scientists interested in gravitational and space biology to share information and join together to speak with a united voice in support of this field of science. The effects of gravity have been acknowledged since Galileo’s time, but only since the 1970s has gravitational research begun to attract attention. With the birth of the space age, the opportunity for experimentation over the full spectrum of gravity finally became a reality, and a new environment and research tool became available to probe gravitational phenomena and expand scientific knowledge. Space and spaceflight introduced new questions about space radiation and the physiological and psychological effects of the artificial environment of spacecraft.

The objectives of ASGSR are:

- To promote research, education, training, and development in the areas of gravitational and space research and to apply the knowledge gained to a better understanding of the effect of gravity and space environmental factors on the flora and fauna of Earth.
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Review articles are typically 10 - 15 pages in length. These manuscripts are often solicited from symposium speakers at the annual ASGSR meeting, but they are not limited to those solicitations. Any author may approach the editorial board with a suggestion or request to submit a review article, which will be peer-reviewed as any other paper.
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- **Text**: Single spaced, Times New Roman 11, paragraphs indent 0.25 inch. Do not insert line space after paragraph. Do not use page breaks.
- **Citations**: In text by (Author, year) mode; details below.
- **Headings**: HEADING LEVEL ONE; Bold, all capital.
- **Subheadings**: Heading level two; Bold, title case.
- **Subheadings**: Heading level three; Italics, non-bold, sentence case.
- **Reference section**: Must comply with format below, each citation separated by line space.

Length

Length is dependent on the type of manuscript being submitted; see overviews above for specifics. One page of a GSR article is typically comprised of 750 – 950 words (depending on the number of figures) and articles range from 2-3 pages for Short Communications, to 15 pages for full Research Papers and Review Articles.

Abbreviations

- Do not use abbreviations other than those that are standard for international usage.
- Use SI units as far as possible.
- Use g (italicized) for unit gravity, to distinguish it from the standard abbreviation g (not italicized) for gram.
- Any acronyms that are used in the manuscript must be defined at first mention.

Arrangement

Arrange the manuscript in the following order, with all pages numbered consecutively in the footer of the lower right corner. The last name of the first author should precede each page number. A Template in Microsoft Word can be downloaded from the Journal website (http://GravitationalAndSpaceResearch.org) that conforms to the general Journal requirements.

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Keywords: List of 3 to 10 keywords

Body of paper: For Research Papers, the body of the paper should be arranged into subsections for Introduction, Materials and Methods, Results, and Discussion. Review Papers should be organized in a manner appropriate to the subject. Methods papers should include a short Introduction and also a Discussion of the application addressing the significance of the method being described.

References and Citations: Cite each reference in the text by author(s) name(s) and the publication date: Examples: Smith, 1989 (one author); Smith and Jones, 2001 (two authors); Smith et al., 2010 (more than two authors). An EndNote® Style file can be downloaded at the GSR website (http://GravitationalAndSpaceResearch.org).

- Alphabetize the reference list by authors' last names.
- List only published or in-press articles. Unpublished results, including personal communications and submitted manuscripts, should be cited as such in the text.
- References formatted as follows: last name(s) of author(s), followed by initials with no space; year of publication in parentheses; article title in sentence case, followed by a period; journal title (unabbreviated and italicized), followed by volume number in bold, issue number in parenthesis (if applicable), a colon, a space, and page numbers.

Four examples below:

Journal Article:

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Figure legends: Provide full, descriptive figure legends for each figure; each figure legend should be composed of a short descriptive title, followed by a description of the material illustrated in the figure. Figure legends should provide any information key to understanding the material presented in the figure.

Figures: Submit Figures as individual graphics files (TIF, BMP, or high resolution JPG). Resolution must be at least 300dpi for photographs and 600dpi for line graphics. Include Figure number in the file name when uploaded. For publication, Figures will be formatted to either one column width (3 inches / 7.6cm) or across two columns (6.3 inches / 16cm). Note that all lettering and numbers within Figures must be at least font size 8 when reduced to publication width. This restriction includes numbers and axis information in graphs as well. Authors are encouraged to test size their Figures in these two dimensions to ensure compliance, as Figures which contain illegible annotations will be returned for
reconfiguration.

- Number Figures consecutively as they are used in the text. Use Figure 1, i.e., capitalize and the full word “Figure” (do not use Fig. 1).
- The first time a Figure is discussed, refer to it actively rather than parenthetically.
- Provide enough information in the Figure Legend such that the reader can understand the Figure without significant input from the text.
- Designate Figure sections with letters and explain all symbols and abbreviations that are used in the Figure.

**Tables:** Provide at the end of the manuscript.

- Number Tables consecutively as they are used in the text.
- The first time a Table is discussed refer to it actively, rather than parenthetically.
- Give each Table a concise title, followed by a legend that makes the general meaning of the Table comprehensible without reference to the text.
- Tables should be constructed in Word or Excel with the general format below. Font size 10 should be used for the primary Table text, but font size 8 may be used for footnotes or annotations.

| Table 1. Atmospheric pressure relative to altitude |
|------------------|------------------|------------------|
| Pressure (kPa)   | Altitude (m)     | Comments                  |
| 101 – 70         | 0 – 3000         | tropical / temperate / taiga biome - many examples of human habitation |
| 70 – 50          | 3000 – 5500      | tundra / alpine biome - few examples of human habitation |
| 50 – 30          | 5500 – 9000      | extreme terrestrial elevations - humans require supplemental oxygen |
| 30 – 5           | 9000 – 27000     | plants can survive as long as temperature is mediated and water is available |

Footnotes as necessary

**Manuscript Peer Review and Preparation of Final Version**

Prior to publication, manuscripts are reviewed by the managing editor who is assigned to an author’s article, and by two to three external scientific reviewers. Manuscripts submitted without complying with submission requirements may be returned for format changes before being accepted for review.

Once a manuscript is accepted, the manuscript is reviewed internally for copyediting, and then sent to the publishing editor. Page proofs are provided to the authors for review prior to publication.

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- Past or present association as thesis advisor or thesis student.
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**Basic elements of informed consent:**

In seeking informed consent the following information shall be provided to each subject:

- A statement that the study involves research, an explanation of the purposes of the research, and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental;
- A description of any reasonably foreseeable risks or discomforts to the subject;
- A description of any benefits to the subject or to others which may reasonably be expected from the research;
- A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject;
- A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained;
- For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained;
- An explanation of whom to contact for answers to pertinent questions about the research and research subjects' rights, and whom to contact in the event of a research-related injury to the subject; and
- A statement that participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.
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Clinorotation Affects Induction of the Heat Shock Response in *Arabidopsis thaliana* Seedlings

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**ABSTRACT**

Clinorotation used to simulate microgravity effects in ground-based experiments is considered as a mild stress factor for plants. We have assumed that it might influence the plant tolerance to other stressful factors. To test this, *Arabidopsis thaliana* seedlings were grown on a horizontal clinostat (2 rpm) or under stationary conditions (control), and then were subjected to heat treatment. The kinetics of gene expression of cytosolic HSP70s and HSP90s during exposure to 37°C for 0.5-2 h was examined by RT-qPCR to estimate level of the heat shock reaction. It was shown that clinorotation caused the minor increase in transcript abundance of five AtHSP70s and AtHSP90-1 under normal temperature, as well as a faster onset and enhancement of their induction during heat shock. The heat tolerance was evaluated as a function of seedling survival after exposure to 45°C for 45 min. Seedlings grown under clinorotation were determined to withstand heat treatment better than seedlings grown under stationary conditions. The obtained data support the assumption that clinorotation may provide cross-protection of plants against fluctuations in environmental conditions.

**INTRODUCTION**

Steady growing of plants as an autotrophic link of Life Support Systems is one of the key issues for long-term manned space missions (Ferl et al., 2002; Wheeler, 2010). Their ability to cope with spaceflight environments is thus of critical importance. It has been shown that microgravity, an unusual factor for plants, does not prevent their growth and development but causes changes in structural and functional organization of cells, and is considered as a mild stressor (Medina et al., 2011; Kordyum, 2014). However, its influence on the development of adaptive reaction to other unfavorable factors is not well understood. To study effects of reduced gravity in plants in ground-based experiments, we used clinorotation. This study is focused on how clinorotation might influence a conserved reaction of cells to elevated temperatures, known as the heat shock response. A key component of this response is upregulation of heat shock proteins (HSP) (Vierling, 1991; Sørensen et al., 2003; Wang et al., 2004). Functioning as molecular chaperones, they protect and reactivate protein structures affected by stress events.

It has been found that spaceflight factors, including microgravity, activate HSP expression in plant cells (Paul et al., 2005, 2012; Zupanska et al., 2013; Hausmann et al., 2014). In our previous work, some temporary increase in the
HSP70 and HSP90 levels in pea seedlings for the first hours of clinorotation was determined (Kozeko and Kordyum, 2007). In addition, seedlings grown under permanent rotation showed a perceptible increase in HSP induction in response to high temperatures, when compared to seedlings grown motionlessly (Kozeko, 2008).

Numerous investigations have confirmed the importance of HSPs in plant tolerance to heat, as well as to a range of other stressful factors (Vierling, 1991; Sørensen et al., 2003; Wang et al., 2004). Moreover, their involvement in cross-protection between different types of stress has been examined (Banti et al., 2008; Montero-Barrientos et al., 2010).

In the present work, we have studied the influence of clinorotation on gene expression of HSP70 and HSP90 during the following heat shock treatment, as well as thermostolerance in Arabidopsis thaliana seedlings. HSP70s are considered as the principal stress-induced HSP family in many species (Sørensen et al., 2003). HSP90s also respond to damaging factors (Milioni and Hatzopoulos, 1997; Wang et al., 2004). In A. thaliana, there are five homologues of HSP70s (DnaK subfamily) and four homologues of HSP90s that are localized to the cytosol. Among these AtHSP70-4, AtHSP70-5, and AtHSP90-1 are highly induced by heat (Milioni and Hatzopoulos, 1997; Lin et al., 2001; Sung et al., 2001). Refer to Table 1 for gene names and AGI codes.

### Table 1. Primers of target genes used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>AGI code</th>
<th>Primer sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtHSP70-1</td>
<td>AT5G02500</td>
<td>F: AAACCTTAGCCGCCTTTATTC R: GATAGCTGGTCTTCTCTTTAC</td>
</tr>
<tr>
<td>AtHSP70-2</td>
<td>AT5G02490</td>
<td>F: AGCTTGTGAGAGAAAGAG R: ACGGGTGATGGAATAGA</td>
</tr>
<tr>
<td>AtHSP70-3</td>
<td>AT3G09440</td>
<td>F: GACATTAGTGAAAAACCGAGAG R: GTCTGAGCCCGTAGATGACAAAG</td>
</tr>
<tr>
<td>AtHSP70-4</td>
<td>AT3G12580</td>
<td>F: AGGGCACCGAAACAAGGACACAAAC R: TCAGCCGACACATTCAAGGATACCA</td>
</tr>
<tr>
<td>AtHSP70-5</td>
<td>AT1G16030</td>
<td>F: GGAGCCTATCTCTGGCTTTATG R: GGCTCTCGTACCCCTCTTATC</td>
</tr>
<tr>
<td>AtHSP90-1</td>
<td>AT5G52640</td>
<td>F: GTTACCCCTATCTACCTTTGGACCG R: CTGATTGTTGATGAGTCCAC</td>
</tr>
<tr>
<td>AtHSP90-2</td>
<td>AT5G56030</td>
<td>To detect mRNA of three genes in total:</td>
</tr>
<tr>
<td>AtHSP90-3</td>
<td>AT5G56010</td>
<td>F: GCTACCCAATCTCTCTGGATT R: GTACTCCTCCTTGTTGATCTC</td>
</tr>
<tr>
<td>AtHSP90-4</td>
<td>AT5G56000</td>
<td>R: GTACCTCTCCTGTGGATCTC</td>
</tr>
<tr>
<td>AtUBQ5</td>
<td>AT3G62250</td>
<td>F: AACCGCTTGGAGATGATCAtCGGTTACG</td>
</tr>
</tbody>
</table>

### MATERIALS AND METHODS

#### Plant Material, Growth Conditions, and Heat Treatments

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds were sterilized with 70% ethanol and hypochlorite solution (3% Cl), washed five times with sterile water, incubated in the dark at 4°C for 2 d, and plated with equal spacing on germination medium (0.5 Murashige and Skoog mineral salt complex, 1% sucrose, 0.8% agar) in Petri plates. Seeds were allowed to germinate and grow on a horizontal clinostat (2 rpm) or under stationary conditions (control) at 22 ± 1°C, photoperiod of 16 h light/8 h dark, and light intensity ~110 μmol m⁻²s⁻¹. After 12 d of growth, rotated and control seedlings were subjected to heat treatments.

For seedling survival assay, plates with 12-d-old seedlings, grown as described above, were
exposed to 45°C for 45 min. Following the heat treatment, seedlings were returned to the stationary conditions and their survival was recorded for the next 6 d of incubation. The number of viable seedlings that were still green and generated new leaves was quantified to determine the survival rate. This assay was also performed for 5-d-old seedlings.

For analysis of gene expression, 12-d-old seedlings grown, as described above, were exposed at 37°C for 0.5, 1, and 2 h and then frozen at -70°C. Three independent experiments were performed for each experimental condition.

**RT-qPCR**

Total RNA was extracted from seedlings using innuPREP Plant RNA Kit (Analytik Jena). 1 μg of each sample was reverse transcribed into cDNA using RevertAid First Strand cDNA synthesis Kit (ThermoSci) with oligo(dT)18 primer. Real-time amplification was performed using the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific) and the iQ5 amplifier (Bio-Rad). The following PCR program was employed: 95°C for 2 min, then 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 40 s. UBQ5 was used as the reference gene for all assays. Primer pairs, as outlined in Table 1, were designed using IDT PrimerQuest Tool (https://eu.idtdna.com/Primerquest/Home/Index). The conserved sequences of highly homologous AtHSP90-2, AtHSP90-3, and AtHSP90-4 were used to design the common primers for them. Specificity of each primer pair and quality of RT-qPCR products was checked by melting curve analysis and agarose gel electrophoresis. Quantity of each transcript was measured in three independent biological samples with three analytical replicates. UBQ5 expression was used to normalize the transcript levels in each sample. Relative expression of each gene was calculated by the comparative ΔΔCt method relatively to the transcript level of the control at 22°C.

**Data Analysis and Statistics**

For data analysis, the mean and standard deviation were calculated for each group. A two-way ANOVA was used to determine significant differences for the effects of clinostat rotation and high temperature on transcript levels (p < 0.05). For the seedling survival and effect of clinostat rotation on HSP expression at normal temperature, the means for the control and experimental groups were compared using Student’s t-test (p < 0.05).

**RESULTS**

In order to test whether clinorotation influences the heat shock response, seedlings grown under stationary conditions (control) or clinostat rotation for 12 d were exposed to 37°C for 0.5-2 h. Levels of mRNAs of cytosolic HSP70s and HSP90s in seedlings were quantified using RT-qPCR. We used one set of primers for AtHSP90-2, AtHSP90-3, and AtHSP90-4, since their sequences are highly homologous and we could not synthesize specific primers for each gene. In addition, these HSP90s are known to show similar pattern of gene expression at both normal and high temperatures (Milioni and Hatzopoulos, 1997). Therefore, we measured the total mRNA level of these three genes to characterize gene expression of constitutive cytosolic HSP90s.

Figure 1 shows that exposure to 37°C resulted in rapid induction of HSP genes in control and rotated seedlings, but with differences in magnitudes of their expression. The mRNA level of constitutive AtHSP70-1 steadily increased at a low rate over the duration of heat exposure, and only a tendency to its enhancement after clinorotation was observed. Induction of constitutive AtHSP70-2, AtHSP70-3, as well as AtHSP90-2, AtHSP90-3, and AtHSP90-4 (in total) reached the maximum levels at 1 h of heat treatment, while thereafter the mRNA amounts declined. At the same time, a higher expression rate for AtHSP70-4 and AtHSP70-5, and, conversely, a tendency to decline for expression of HSP90s were determined in rotated seedlings, when compared to control ones. Inducible AtHSP70-4, AtHSP70-5, and AtHSP90-1 showed strong induction by 30 min of heat treatment, its significant increase within the next 30 min and a steady high level for the second hour. After clinorotation, the induction of these genes occurred faster and reached a higher level, especially for AtHSP70-4 and AtHSP70-5.

As illustrated in Figure 2, comparison of the transcript abundance in rotated and control seedlings at 22 ± 1°C (0 h of heat exposure) showed an increase of 2- to 7-fold under clinorotation, with the exception of constitutive HSP90s. The heat tolerance of seedlings was evaluated as a function of their survival after an
Figure 1. An effect of clinostat rotation on induction of HSP70 and HSP90 genes in Arabidopsis thaliana (Col) seedlings during heat shock. 12-d-old seedlings grown under stationary conditions (control) or clinorotation were exposed at 37°C for the indicated times. Expression levels were assessed using RT-qPCR, and normalized with respect to UBQ5 mRNA. Relative mRNA amounts were calculated as a fold change to the control (= 0 h of the heat treatment). Data are means of three independent biological samples with three analytical replicates ± s.d. The effect of high temperature was significant for all the HSPs; the effect of clinorotation was significant for AtHSP70-2, AtHSP70-3, AtHSP70-4, AtHSP70-5, and AtHSP90-1 (Two-way ANOVA, p < 0.05).
Figure 2. An effect of clinostat rotation on expression of HSP70 and HSP90 genes in Arabidopsis thaliana (Col) seedlings. The bars present the fold change of transcript abundance in 12-d-old seedlings grown under clinorotation relative to the control (seedlings grown under stationary conditions, = 1; dotted line). HSP90 c represents AtHSP90-2, AtHSP90-3, and AtHSP90-4 (in total). Bars denoted with an asterisk (*) indicate differences between the rotated and control seedlings that are statistically significant, compared with the control (Student’s t-test, p < 0.05). Expression levels were assessed using RT-qPCR and normalized with respect to UBQ5 mRNA. Data are means of three independent biological samples with three analytical replicates ± s.d.

Figure 3. An effect of clinostat rotation on heat shock survival of Arabidopsis thaliana (Col) seedlings. 12-d-old seedlings grown under stationary conditions (control) (A, B) or clinorotation (C, D) at 22 ± 1°C were exposed at 45°C for 45 min and recovered under the stationary conditions. (A, C) seedlings before the heat treatment and (B, D) seedlings after a 6-d recovery period (B, D). (E) Survival rate of the seedlings after the heat treatment. Data are means ± s.d. (n = 3 plates with ~25 seedlings each; * Student’s t-test, p < 0.05).

exposure to 45°C for 45 min. This temperature is specified as a standard to detect alterations in A. thaliana plant thermotolerance (Silva-Correia et al., 2014). Figure 3 depicts that different survival rates were observed in heat-treated seedlings grown under clinorotation compared with stationary conditions. A portion of rotated seedlings were able to generate new leaves over
the 6-d post-exposure period, whereas control seedlings showed almost complete death. Similar results were obtained with 5-d-old seedlings as illustrated in Figure 4.

Figure 4. An effect of clinostat rotation on heat shock survival of Arabidopsis thaliana (Col) seedlings. (A) 5-d-old seedlings grown in the stationary conditions or under clinostat rotation at 22 ± 1°C were exposed at 45°C for 45 min, and recovered under the stationary conditions for 3 d. (B) Survival rate of the seedlings after the heat treatment. Data are means ± s.d. (n = 3; * Student’s t-test, p < 0.05).
DISCUSSION

HSPs/chaperones play a role in protecting cells against multiple stressful conditions (Sørensen et al., 2003; Wang et al., 2004). Their upregulation during the heat shock response provides resumption of protein homeostasis and leads to a higher level of thermotolerance (Vierling, 1991). Moreover, an increasing number of studies demonstrate that HSPs contribute to cross-protection between heat stress and other types of stress in plants (Banti et al., 2008; Montero-Barrientos et al., 2010). In this study, we applied heat treatment following clinorotation of seedlings. As previously mentioned, both factors can activate HSP synthesis. The obtained results showed that clinorotation caused the minor increase in transcript abundance of five cytosolic AtHSP70s and AtHSP90-1 under normal temperature, as well as a faster onset and enhancement of their induction during heat shock. Notably, the strongest effect was revealed for inducible AtHSP70-4, AtHSP70-5, and AtHSP90-1, which are especially important for stress tolerance (Sørensen et al., 2003). In contrast, the total expression of constitutive AtHSP90-2, AtHSP90-3, and AtHSP90-4 was not altered under clinorotation and showed less induction by heat treatment in rotated seedlings, when compared to seedlings grown under stationary conditions.

A weak increase in the expression levels of several HSPs in seedlings grown under clinorotation at normal temperature likely reflects adaptation of cells to rotation. It is noteworthy that clinorotation may have multiple impacts on organisms, including not only simulation of the microgravity effects, but also mechanical and electromagnetic influences, etc. However, in our previous work with pea seedlings, comparison of the effects of horizontal and vertical rotation showed small but statistically significant differences in HSP70 and HSP90 levels that could be attributed to simulated microgravity (Kozeko and Kordyum, 2006).

As a whole, the time course of HSP expression during heat exposure was in good agreement with the previous findings by other researchers (Milioni and Hatzopoulos, 1997; Sung et al., 2001). At that, the magnitudes of AtHSP70s and AtHSP90-1 induction were higher in rotated seedlings relative to control ones. These findings correlate with our previous Western-blot data indicating enhanced induction of HSP70 and HSP90 in pea seedlings in response to high temperatures (38-45°C) after long-term clinorotation (Kozeko, 2008). Thus, increasing the heat shock response under the influence of clinorotation was shown at both transcriptional and translational levels. However, the obtained results could indicate two opposite situations. On the one hand, the higher inducibility of several HSPs may reflect higher sensitivity of rotated seedlings to heat shock; on the other hand, it may result from pre-adaptation of seedlings under clinorotation. To test that, the seedling survival assay was carried out. Its results showed that seedlings grown under clinorotation withstood exposure to the near lethal temperature better than seedlings grown under stationary conditions. These data support the assumption that clinorotation may provide cross-protection of plants against fluctuations in environmental conditions, which correlates with the data on HSP expression.

REFERENCES


Research Article

Self-Assembly of Protein Fibrils in Microgravity

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ABSTRACT

Deposits of insoluble protein fibrils in human tissue are associated with amyloidosis and neurodegenerative diseases. Different proteins are involved in each disease; all are soluble in their native conformation in vivo, but by molecular self-assembly, they all form insoluble protein fibril deposits with a similar cross β-sheet structure. This paper reports the results of an experiment in molecular self-assembly carried out in microgravity on the International Space Station (ISS). The Self-Assembly in Biology and the Origin of Life (SABOL) experiment was designed to study the growth of lysozyme fibrils in microgravity. Lysozyme is a model protein that has been shown to replicate the aggregation processes of other amyloid proteins. Here the design and performance of the experimental hardware is described in detail. The flight experiment was carried to the ISS in the Dragon capsule of the SpaceX CRS-5 mission and returned to Earth after 32 days. The lysozyme fibrils formed in microgravity aboard the ISS show a distinctly different morphology compared to fibrils formed in the ground-control (G-C) experiment. The fibrils formed in microgravity are shorter, straighter, and thicker than those formed in the laboratory G-C experiment. For two incubation periods, (2) about 8.5 days and (3) about 14.5 days, the average ISS and G-C fibril diameters are respectively:

<table>
<thead>
<tr>
<th>Period</th>
<th>D_{ISS}</th>
<th>D_{G-C}</th>
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<tbody>
<tr>
<td>2</td>
<td>7.5nm ± 31%</td>
<td>3.4nm ± 31%</td>
</tr>
<tr>
<td>3</td>
<td>6.2nm ± 33%</td>
<td>3.6nm ± 33%</td>
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INTRODUCTION

Deposits of insoluble protein fibrils are known to be associated with amyloidosis and neurodegenerative diseases such as Alzheimer’s,
Several different proteins are involved in the different diseases. All of these so-called amyloid proteins are soluble in their native conformation in vivo, but over time, they all form insoluble protein fibril deposits with a similar cross β-sheet structure (Sunde and Blake, 1997; Jiménez et al., 2002).

Amyloid proteins will also form protein fibril deposits in vitro with the same cross β-sheet structure as in vivo. They self-assemble into fibrils directly from solution when the conditions, such as protein concentration, pH, ion concentration, and temperature are right. Depending on these parameters, different pathways to fibril formation may be followed. Protein monomers either assemble directly into fibrils or follow an intermediate aggregation of oligomers that form before the formation of fibrils (Asgregli and Boden, 2006; Estroff and Hamilton, 2006; Perutz et al., 2002; Hill et al., 2011; Necula et al., 2007; Pellarin and Caflisch, 2006). These different pathways affect fibril diameter, length, and helicity (Woodard et al., 2014; Ward et al., 2012; Dahlgren et al., 2002; Kayed et al., 2003). The study of amyloid fibril formation in vitro helps to inform amyloid disease research.

Figure 1. AFM images of lysozyme aggregation showing advanced stages of the amyloid fibril formation process (Woodard et al., 2014). Left: the merging of fibrils into a helix configuration is indicated by the two arrows. Right: A tangled interlocking network prevents fibrils from rotating and halts helix formation. (Note the different image scales.)
To date, most protein aggregation studies have been carried out in ground-based laboratories. After fibril formation and continued incubation these fibrils interact and form macromolecular structures with mature fibrils interweaving with each other (see Figure 1). This network of fibrils can suspend the solution and convert the surrounding fluid into a gel with increased viscosity and opacity (Terech, 2006). These networks create the building blocks for protein plaques to form (Kodali and Wetzel, 2007; Chiti and Dobson, 2006). Currently the morphology of fibril growth cannot be predicted (Bitan et al., 2005; Kowalewski and Holtzman, 1999).

Understanding the colloidal chemistry and biochemistry of amyloid fibril formation is helped by controlling process parameters, such as manipulation of the solution’s stabilization with ions, changes in temperature, or pH. It may be possible to inhibit or accelerate amyloid fibril formation. For example, increased salt concentration in the initial buffer solution can increase aggregation and gelation rates (Woodard et al., 2014; Hill et al., 2009, 2011; Fujiwara et al., 2003; Wang et al., 1996).

Gravity has an effect on biological processes even at the cellular level (Tabony et al., 2007). Self-assembly and self-organization of molecules into larger macromolecular structures can be affected by the presence of this weak directional external force. In a space-based laboratory gravitational pressure gradients in fluids do not develop, so natural buoyant convection is not present. Microgravity is a realistic environment to emulate cellular conditions, where surface tension effects dominate viscous effects. The fibril growth process must rely on the movement of protein molecules onto and off of the growing fibrils. In microgravity, surface tension and molecular diffusion will be the dominant interactions.

Although life probably arose in a planetary environment, molecular self-assembly is also of interest to the study of the origin of life. The development of cellular activity was dependent upon the creation of large complex molecular structures in the chemical and environmental conditions present when life originated. Experiments carried out in microgravity may therefore lead to a better understanding of the dynamics driving cellular molecular self-assembly process. Florida Tech’s Self-Assembly in Biology and the Origin of Life (SABOL) study demonstrated protein fibril growth in microgravity onboard the International Space Station (ISS) using a novel, autonomous NanoLab. Protein fibrils grown in microgravity demonstrate a morphology significantly different from samples grown on Earth in a nearly identical Ground-Control (G-C) system.

MATERIALS AND METHODS

Buffer/Lysozyme Solution

The buffer/lysozyme solution for the SABOL experiment was chosen to optimize the fibril growth period from onset through gelation of 20 to 30 days, the estimated time in microgravity. The optimal methods for lysozyme preparation, fibril formation, and fibril characterization are well understood (Burnett et al., 2014; Hill et al., 2011; Woodard et al., 2014). The buffer solution was prepared with 10 mM glycine in DI water, titrated to pH 2.5 with HCl. To ensure uniformity across samples, the titration was performed in a large beaker of solution prior to the addition of lysozyme. The final HCl concentration was approximately 36 mM. Lysozyme (BSG, Napa, CA) was then dissolved in the buffer solution at a concentration of 20 mg/mL. Acidic conditions are required and at this pH the lysozyme has a charge of approximately +26. Heating the sample close to the denaturing temperature of 55°C (Hill et al., 2011) is also required. At this temperature lysozyme begins to unfold from its native conformation and increased Brownian motion assists the Van der Waals attractive forces in overcoming the electric repulsive forces leading to aggregation.

Atomic Force Microscope

The samples were imaged with a Molecular Imaging multi-purpose scanner Atomic Force
Microscope (AFM). PicoView version 1.12 AFM software was used, and all images were acquired in contact mode. A Bruker SNL-10 silicon-tip on nitride cantilever was used, and an Olympus IX71 optical microscope was used to center the laser light reflecting off the cantilever. The quadrant photodiode’s position was then adjusted to null the signal. Cantilever probes are typically 600 nm in height with a 10 nm radius of curvature and a force constant of 0.12 N/m. AFM tip with curvature radii greater than 30 nm or damaged were replaced.

A voltage set-point of 3 volts was used, translating to 5.4 nN of load force due to an average of 15 mV/nm deflection sensitivity and 0.12 N/m cantilever elasticity. This set-point was used for the majority of images. Raster speed ranged from 1 to 4 lines/sec, and all of images are 1024 x 1024 pixels. Fields of view (FOV) of 2x2, 5x5, 10x10, and 20x20 µm were the most common. Contact mode, where the probe moves up and down to maintain the constant set point force, was used throughout. Topographic images were used both to determine three-dimensional structure and for dimensional measurements, while deflection images were used to demonstrate shape and texture.

Lysozyme Fibril Deformation

Lysozyme fibrils deform under the force applied by the cantilever. The cantilever load force is determined by the force set-point. A force set-point of 3 volts was used for all AFM images in the SABOL experiment. A calibration was determined by measuring ground-based lysozyme fibril heights for increasing values of the force set-point. Images were taken with the least amount of force first, then increasing until the samples no longer produced readable data. Figure 2 shows height vs. force set-point data for lysozyme fibrils. Each data point is an average of 20 lysozyme fibril height measurements from each force set-point image. The error bars shown are the measurement standard deviations.

Fibril height decreases with increasing load force. A linear fit to this trend, shown by the dashed line, has an R-squared value of 0.93 and a y-intercept of 2.29 nm. All topographic images in this study used a force set-point of 3 volts. Figure 2 can thus be used to correct height measurements of lysozyme fibrils to heights with no load force applied:

\[ h = h_m \times 1.294 \]

where \( h_m \) is the measured height, 1.294 is the calibration constant, and \( h \) is the undeformed height.

![Sample Deformation](image)

**Figure 2.** Deformation of lysozyme samples under different load forces (force set-points). Each data point is an average of 20 lysozyme fibril height measurements made at each force set-point. Error bars are the measurement standard deviations. The dashed line is a linear fit to the data.
Sample Preparation

Samples of the aggregated protein were prepared for AFM imaging as follows: First, both undiluted and diluted slides were prepared to ensure protein fibrils were visible against the flat mica substrate and not covered with excess solution protein. Samples were preserved at 4°C, since freezing causes damage to the fibrils.

Drawing lysozyme gels into a standard pipette resulted in fibril breakage due to the gel’s large viscosity resulting in fluid shear as the gel was drawn through the small orifice of the pipette (Woodard et al., 2014). Consequently, when pipetting fibrils and/or gels, the tips of the pipettes were first cut off to enlarge the orifice diameter to ~1 mm.

To prepare AFM slides, 10 µL of 0.01 N NaOH was applied near the center of a freshly peeled mica slide to precharge the substrate. After two minutes, 10 µL of sample was applied to the substrate at the same location. After an additional 10 minutes, the sample was rinsed with 2 mL of DI water by allowing the water to flow over the substrate to remove salts and unbound proteins. The water was drained and any droplets adhering to the substrate were removed with a tangentially applied soft jet of nitrogen gas. The slide was then dried in an oven set to 55°C for 2 hours.

SABOL Hardware for the International Space Station Experiment

The Self Assembly in Biology and the Origin of Life (SABOL) experiment was designed to study the self-assembly of lysozyme fibrils in microgravity. An exploded view of the hardware is shown in Figure 3. The experiment is housed in a 1U NanoRacks chassis, measuring 10x10x15 cm. There are 9 vials arranged in a 3x3 array. Each vial has linear stepper motor actuation mechanism, to introduce protein powder into the buffer solution, an individual heater and thermal control system. There is a custom aluminum shell, a support structure for the vials, and a USB connector for power. There are two printed circuit boards (PCB). The side PCB is used to measure and control the temperature of each vial individually and perform data acquisition. The top PCB contains the components needed to operate the actuation mechanisms at the appropriate times. Time is based on an internal battery powered timer to protect against ISS main power loss events.

The fully assembled SABOL hardware with the outer cover removed is shown in Figure 4. All 9 linear stepper motors can be seen above the vial support structure and 3 of the insulated vials can be seen in Figure 4.

Figure 3. An exploded view of the SABOL experiment. The hardware was designed to fit within the volume, mass, and power constraints of a 1U NanoLab module. There are 9 independently operated vials used to provide a range of incubation times covering the growth phase of lysozyme fibrils.
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Figure 4. Fully assembled SABOL NanoLab with samples loaded. Unit shown just before the outer shell was installed.

Actuation mechanism

An important design element of the SABOL experiment was to ensure that the lysozyme powder remained separated from the buffer solution until the incubation period in orbit is initiated. Furthermore, the vials must maintain a seal at all times and not allow air bubbles to form. The vials and all of their internal components are made out of Polypropylene. Two views of a vial are shown in cross-section in Figure 5. The image on the left shows a vial in the unactuated configuration; the image on the right shows it in the actuated configuration. Each vial has two separate compartments initially isolated from each other. One compartment, containing the buffer solution, consists of a 22 mm diameter tube with a volume of 2.5 mL, bounded on the bottom by the floating piston and on the top by the loading piston. The other compartment consists of the filling slot on the side of the loading piston, with just enough volume to hold 0.05 g of lysozyme powder. The loading piston has a threaded hole in its top that is attached to the shaft of a linear stepper motor. Actuation occurs when the stepper motor drives the loading piston down, uncovering the filling slot and bringing the lysozyme powder into contact with the buffer. The floating piston reacts back to maintain a constant volume.

Thermal control system

Each vial has an independent thermal control system consisting of Nichrome heater wire wrapped around each vial, a layer of insulation over the heater wire, and a thermocouple bonded into a capped hole in the bottom of the floating piston. The control system consists of a simple set-point regulation system $T = T_o \pm \Delta T$ originally chosen as $T_o=55^\circ C$ and $\Delta T=1.5^\circ C$. The 5 Watts of electrical power available to the 1U module through its USB connector was only sufficient to run 4 of the heaters at once, so a heating schedule was developed where no more than 4 heaters were on at a time. This allowed incubation times to cover from 3 to 27 days in increments of 3 days. One vial remained unheated to serve as a control.

The start of an incubation period for a given vial is implemented as follows: first all heaters are turned off to provide enough power to run a stepper motor. The stepper motor of the vial to be actuated is turned on, run to completion, and then turned off. That vial’s heater is then turned on and heaters for the other vials still within their incubation periods are turned back on. When the
time is reached for a vial’s incubation period to end, its heater is turned off. Since the heating and cooling time constants for the vials are nearly an hour, and the incubation time periods are days, the few minutes it takes to execute the vial actuation has negligible effect on the incubation. The incubation time period for a given vial is determined as the time the temperature exceeds 50°C.

Figure 5. Cross-section of polypropylene vials before and after actuation.

Ground-Control NanoLab

An identical Ground-Control (G-C) NanoLab was built so that it could be run on ground with the same timing protocols and environmental conditions as the ISS NanoLab, except for the effect of microgravity.

Preparation and flight

The day before the experiment was handed over to NanoRacks, samples were loaded as follows: With the bushing and loading piston removed, each vial was filled with 2.5 mL of buffer solution and the floating piston was pushed up, bringing the fluid level up to the top of the vial. Then dry lysozyme protein powder was placed in the open slot of the loading piston. The loading piston was placed inside the bushing, sealing off the protein powder. The bushing with the loading piston installed was then placed at the top of the vial and inserted in a manner that sealed the buffer solution with no air bubbles. The complete vial assembly was then threaded onto the stepper motor shaft. This was repeated for each of the nine vials.

The SABOL experiment was carried to the International Space Station (ISS) in the Dragon capsule of the SpaceX CRS-5 mission and returned to Earth in the same Dragon capsule after 32 days. Table 1 gives the duration times for both the ISS and G-C NanoLab’s. The G-C NanoLab was run for the same amount of time as the ISS NanoLab. No telemetry was available from either
to monitor their progress. The ISS NanoLab was kept near 4°C from deorbit throughout splashdown, retrieval, and transportation back to Florida Tech.

Table 1. NanoLab Timeline (EST).

<table>
<thead>
<tr>
<th></th>
<th>Plugged In</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>ISS NanoLab</td>
<td>Jan. 13th 1:22 PM</td>
<td>25d 18h 28m</td>
</tr>
<tr>
<td>G-C NanoLab</td>
<td>Feb. 10th 4:41 PM</td>
<td>25d 18h 28m</td>
</tr>
</tbody>
</table>

**ISS NanoLab post-flight handling**

The ISS NanoLab memory chip, containing temperature data, was accessible after removal of the experiment’s outer shell. Figure 6 shows temperature as a function of time for all 9 vials. Vial 1 was a control that was not heated. The unheated or ambient temperature within the NanoLab, as indicated by vial 1, remained between 33°C and 35°C for the duration of the experiment. Previous testing showed that fibril growth would not start until the temperature was greater than 40°C. Vials 2 through 9 show proper heating for different amounts of time. As one vial turned off another vial actuated the protein into the buffer solution. Then that vial was heated to the incubation temperature.

Next, the four bolts holding the top and bottom support structure together were removed. All 9 vials were unscrewed from their stepper motors, being careful not to disturb the position of the loading pistons. Each of the nine vials were placed in individual plastic bags labeled with the vial’s position and stored in a 4°C refrigerator. The top support could then be examined to see if the stepper motors had fully actuated their respective loading pistons. Figure 7 shows that only 3 of the stepper motor shafts had extended, so only 3 of the vials had successfully actuated while in orbit aboard the ISS. They were vials 5, 6, and 8.

The vial 5 heater was turned on at the beginning of the experiment and turned off at day 15. The temperature remained at about 53°C for the duration of its operation. It dropped to 37°C when turned off and decayed to 36°C by the end of the experiment. Vial 8 heater turned on at day 18. The temperature remained at about 51°C until the experiment was terminated at day 25.75. The vial 6 heater was turned on at day 24. It remained at 51°C until the experiment was terminated at day 25.75. Thus, these three vials provide samples with incubation times (Vial: Time) as follows: (5: 15 days), (8: 7.75 days), (6: 1.75 days).

The loading pistons were extracted from each vial, samples were collected with a 200 µL pipette, and samples were prepared for imaging. The pre-extraction positions of the loading pistons confirmed that only vials 5, 6, and 8 had actuated properly. Also, it was noted that the loading piston slots of vials 5 and 8 had solution remaining in them, and a smooth layer of fluid flush with the outside of the slot. The solution within these slots appeared more viscous than the majority of the solution inside the vial. A sample of solution from the loading piston slot was taken for vials 5 and 8.

**G-C NanoLab handling**

The Ground-Control experiment had some unintended differences from the ISS experiment. The G-C NanoLab contained only 8 vials, as one was damaged during testing. To compensate for this, a thermocouple was suspended in the position of vial 1 to measure ambient air temperature. After completion of assembly and sample loading, the G-C NanoLab was plugged into a USB converter of an uninterrupted power supply (UPS) at Florida Tech. The G-C NanoLab was powered on for the same length of time as the ISS NanoLab, as shown in Table 1. The G-C NanoLab was then cooled to 4°C and kept at that temperature for 6 days to match the conditions of the ISS NanoLab. The G-C NanoLab was then prepared for imaging in the same way as the ISS.
Figure 6. ISS Vial Temperature verses Time. Temperature profiles for each of the ISS NanoLab vials show the time when each heater was turned on, raising the temperature to within the aggregation range (Hill et al., 2009; Woodard et al., 2014), held there for its incubation period, and then turned off.
NanoLab. There was no viscous solution in the loading piston slots as seen in the ISS vials.

Figure 8 shows temperature as a function of time for all vials of the G-C NanoLab. The actuation and heating times were accidently reversed relative to the ISS NanoLab; the shorter incubation times were first on the G-C NanoLab, whereas they were last for the ISS NanoLab. When the experiment was stopped 1.25 days before the planned stop at day 27, this had the unintended effect of losing 1.25 days from different incubation intervals of the ISS and G-C data.

Most of the vials in the G-C NanoLab, except vial 7, reached the incubation temperature. The ambient air temperature inside the G-C NanoLab was between 30 °C and 34 °C. For comparison with the ISS NanoLab, the following G-C NanoLab vials with the similar incubation times can be used — (9: 13.75 days), (4: 9 days), (2: 3 days). The time scale for changes in fibril growth is a few days so this difference between ISS and G-C samples is still useful.

RESULTS AND DISCUSSION

Slides for AFM imaging were prepared, as discussed earlier, using solution taken from the three ISS NanoLab vials that actuated properly: vials 5, 6, and 8. Figure 9 shows an AFM image of solution taken from ISS NanoLab vial 8. This sample was incubated in microgravity for 7.75 days at 51 °C. The image was created with a 5 x 5 µm FOV. There is a clear difference between the lysozyme fibrils seen here and those routinely seen in samples formed in ground-based laboratories. In Figure 1, for example, fibrils formed in microgravity on the ISS are shorter, straighter, and thicker (larger diameter) than samples formed in the laboratory.
Figure 8. Temperature profile of each vial within the G-C NanoLab. The graph shows the time when each heater was turned on, raising the temperature to within the aggregation range (Hill et al., 2009; Woodard et al., 2014). The bold lines represent the temperature profiles from the vials that incubated for periods of time close to the 3 vials that fully actuated in the ISS NanoLab.
Fibrils formed in ground-based laboratories normally use a significantly different vial shape and protein powder mixing system. To determine if the change in fibril morphology could be attributed to the more complex process of actuation or the significantly different geometry of the ISS and G-C NanoLab vials, a complete set of slides for AFM imaging were prepared using solution taken from the three G-C NanoLab vials with closely matching incubation times: vials 2, 4, and 9. Although not ideal, the difference in incubation times of 1.25 days is still a useful comparison since the time scale for significant change in fibril growth is on the order of days. Also, in microgravity, fluid motion by surface tension is likely to pay a large role so the comparison of fibril growth in identical vials is important. Remember the more viscous fluid present in two of the loading slots of the ISS NanoLab.

Another difference between the ISS and G-C NanoLabs is the external forces due to launch, re-entry, and landing experienced only by the ISS NanoLab. During launch the buffer solution and protein powder were in separate chambers so this should have no effect on fibril growth. Also the re-entry and splashdown forces should have no effect since already grown fibrils are stable. Furthermore, the ISS NanoLab was in a transfer bag that included bubble wrap to dampen vibration and the temperature of the ISS NanoLab was held well below the aggregation temperature.

Figure 10 shows representative images from each of the three incubation groups. The images on the left are of ISS formed fibrils, those on the right are of G-C formed fibrils. The incubation time in days is shown for each image. This set of images shows a clear morphological difference between fibrils formed in microgravity and those formed in the ground-control unit. For the first group, the ISS images show no fibril formation but numerous small isolated structures while the G-C images show fully formed fibrils already longer than the 5 µm FOV. For the second incubation group, the ISS fibrils have formed but they are relatively straight and short (significantly

Figure 9. AFM image of solution from ISS vial 8, 7.75 days of incubation, 5 x 5 µm FOV.
less than the 5 µm FOV), whereas the G-C fibrils are fully formed, mature fibrils. For the third incubation group, the ISS fibrils show continued growth with some fibrils almost as long as the 5 µm FOV and maybe a few more complex structures forming; the G-C fibrils show continued formation of long thin complex structures.

Figure 10. Lysozyme fibrils formed in microgravity on the ISS versus lysozyme fibrils formed in the G-C unit under the effects of gravity. All images have a 5 µm FOV.

One difference in the fibril structure present in these images is that the microgravity formed fibrils consistently appear about twice as thick as the G-C formed fibrils. To quantify this difference, the height and width of many fibrils was measured for several images from each ISS and G-C incubation groups. Using the PicoView v1.12 software, a trace was made perpendicular to every accessible fibril at 3 separate places on each fibril. The height and width were determined from the trace. All together there were 18 ISS images used, giving 513 fibril measurements, and 14 G-C images used, giving 232 fibril measurements.

The height measurements can be directly related to fibril diameter using the deformation calibration determined earlier. The width measurements are less reliable since the radius of curvature of the AFM tip is approximately 10 nm, considerably larger than the width of the fibrils.
Figure 11. Heights of protein fibrils formed in microgravity (ISS) compared to protein fibrils formed in a Ground-Control (G-C) experiment.
Figure 11 shows a frequency distribution plot of fibril heights for each of the three incubation periods. The upper figure is for the first incubation period. The average heights of the ISS and G-C fibrils are $H_{\text{ISS}} = 2.2 \pm 0.8 \text{ nm}$ and $H_{\text{G,C}} = 2.3 \pm 1.0 \text{ nm}$, respectively. The average height and standard deviation are calculated directly from the data, not the gamma-fit trend line. At this incubation period the heights are the same within the experimental error. It should be noted however that at this stage the ISS images showed only small isolated structures and no fibrils.

The middle figure is for the second incubation period. The average heights of the ISS and the G-C fibrils are $H_{\text{ISS}} = 5.8 \pm 1.8 \text{ nm}$ and $H_{\text{G,C}} = 2.6 \pm 0.8 \text{ nm}$, respectively. This shows that ISS fibrils have about twice the diameter as G-C fibrils.

The lower figure is for the third incubation period. The average heights of the ISS and G-C fibrils are $H_{\text{ISS}} = 4.8 \pm 1.6 \text{ nm}$ and $H_{\text{G,C}} = 2.7 \pm 0.9 \text{ nm}$, respectively. Again, the ISS fibrils have about twice the diameter of the G-C fibrils.

Another characteristic of these data is that, for incubation groups 2 and 3, the frequency distribution for the ISS grown fibrils is significantly broader than the G-C grown fibrils. This could indicate an actual physical difference, with the microgravity formed fibril heights being more variable than the G-C formed fibrils, or it could be that the measured errors in fibril heights are proportional to the fibril heights. In the latter case, the fractional error for the ISS and G-C measurements should be the same in each incubation group. The fractional errors determined from the data above are: incubation group 2, $H_{\text{ISS}} = 5.8 \text{ nm} \pm 31\%$ and $H_{\text{G,C}} = 2.6 \text{ nm} \pm 31\%$ and for incubation group 3, $H_{\text{ISS}} = 4.8 \text{ nm} \pm 33\%$ and $H_{\text{G,C}} = 2.7 \text{ nm} \pm 33\%$. So, it appears that errors in fibril height measurements are proportional to fibril heights. This also indicates that the height-to-diameter calibration determined with 2.7 nm diameter fibrils is applicable to ~5 nm fibrils as well. Applying the calibration to these data gives the average fibril diameters as:

Incubation Group 2: $D_{\text{ISS}} = 7.5 \text{ nm} \pm 31\%$, and $D_{\text{G,C}} = 3.4 \text{ nm} \pm 31\%$

Incubation Group 3: $D_{\text{ISS}} = 6.2 \text{ nm} \pm 33\%$, and $D_{\text{G,C}} = 3.6 \text{ nm} \pm 33\%$.

CONCLUSION

The primary objectives of the SABOL experiment were to grow protein fibrils in microgravity onboard the ISS and to develop the robust capability of conducting protein fibril research on the ISS. Both of these objectives were met. Lysozyme fibrils were grown in microgravity and the results indicate that this is a fruitful environment for the study of this molecular self-assembly process. Two identical sets of hardware were constructed providing a flight unit and a ground-control unit for future research. Both systems performed well. The only major anomaly in the experiment operation was that, for the flight unit, only 3 of the 9 vial actuation mechanisms operated properly. This can be corrected with a small change in the vial design allowing for a linear stepper motor with more force for any future experiments. This hardware can also be modified and used to study other amyloid protein fibrils.

ACKNOWLEDGMENTS

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Levels of Acid Sphingomyelinase (ASM) in Caenorhabditis elegans in Microgravity

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ABSTRACT

Both Amyotrophic Lateral Sclerosis (ALS) patients and astronauts in spaceflight suffer from muscle atrophy. Previous research suggests that the enzyme acid sphingomyelinase (ASM) may be involved in the pathogenesis of ALS, but it is not known if ASM influences muscle atrophy in microgravity. In this study, Caenorhabditis elegans (C. elegans) were exposed to microgravity conditions on the International Space Station (ISS) within the confines of a Fluid Mixing Enclosure (FME). Return of the FME yielded 72,050 live nematodes, the first demonstration of C. elegans survival of space travel in an FME. After the nematodes returned to Earth, in much larger numbers than seen in previous FME experiments, the size and ASM expression levels in experimental worms were compared to control Earth-bound worms. C. elegans that returned from the ISS were larger in both length and cross-sectional area than the control worms, and they exhibited decreased expression of ASM-1 and ASM-2 proteins. Further research must be conducted to elucidate the role of ASM in muscle atrophy, as there were many limitations to this study. Understanding the role of ASM in muscle atrophy may lead to the discovery of novel targets for treatment of both ALS and muscle atrophy in microgravity. This study was a student led initiative and undertaken as a project within the Student Spaceflight Experiments Program (SSEP), under the auspices of the National Center for Earth and Space Science Education and the Arthur C. Clarke Institute for Space Education.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by a loss of motor neurons and, as a result, skeletal muscle atrophy (van Es et al., 2017). Muscle atrophy is the decrease in muscle size/mass that results from loss of contractile and structural proteins and muscle cells (Brooks and Myburgh, 2014; Fanzani

Key words: Muscle Atrophy; Student Spaceflight Experiments Program (SSEP); Fluid Mixing Enclosure System (FME); International Space Station (ISS); C. elegans; Sphingomyelinase; Amyotrophic Lateral Sclerosis (ALS)

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et al., 2012). The progressive motor neuron loss, denervation-induced and primary muscle dysfunction and wasting in ALS culminates in paralysis and death (van Es et al., 2017; Loeffler et al., 2016). Unfortunately, there are currently no effective curative treatments or therapeutic strategies for ALS (van Es et al., 2017).

Muscle atrophy is prevalent across many chronic diseases (Powers et al., 2016a), in individuals subjected to prolonged bedrest and immobility (Brooks and Myburgh, 2014), and in astronauts in spaceflight (Fitts et al., 2010). In fact, the neuromuscular system is seen as one of the most heavily impacted physiological systems in microgravity (Fitts et al., 2001). Muscle atrophy in microgravity has been previously linked to a lack of exercise opportunities (Fitts et al., 2010). However, the use of resistive exercise countermeasures programs by astronauts was only able to partially mitigate muscle atrophy in microgravity (Fitts et al., 2010; Tanaka et al., 2017). Given the engineering challenge to generate artificial gravity in spaceflight, and the inability of resistance exercise to prevent atrophy in microgravity, it is essential to continue to delineate the molecular mechanisms underpinning muscle loss in space to enable the development of effective interventions to preserve and/or restore muscle mass. We were provided an opportunity by the Student Spaceflight Experiments Program (SSEP), a space education program aimed at students from elementary school to college level administered through the National Center for Earth and Space Science Education (NCESSE) and the Arthur C. Clarke Institute for Space Education, to conduct an experiment on the International Space Station (ISS). We decided to investigate the underlying causes of muscle atrophy in microgravity. This research may bring us one step closer to developing an effective treatment for muscle wasting and weakness in both astronauts during spaceflight and in individuals with chronic diseases such as ALS.

Oxidative stress contributes to the development of skeletal muscle atrophy in both chronic disease states and inactivity via a myriad of cellular signaling networks (Cutler et al., 2002; Powers et al., 2016b). Microgravity has been shown to induce oxidative stress (Takahashi et al., 2017). Sphingomyelin and ceramide accumulation have been linked to both ALS pathogenesis and to oxidative stress (Cutler et al., 2002). Furthermore, in the SOD^{G86R} ALS mouse model, there is evidence of increased gene expression of some sphingomyelinasesenzymes that catalyzes the hydrolysis of sphingomyelin to phosphorylcholine and ceramide (Henriques et al., 2018). This could contribute further to ceramide accumulation. Acid sphingomyelinase (ASM) levels are elevated and activity is increased in the presence of oxidative stress, infection, and inflammation (Kornhuber et al., 2015). However, ASM also releases ceramide, which in itself induces oxidative stress (Jana et al., 2009). These data together suggest that ASM dysregulation may be involved in the pathology of ALS and muscle wasting of spaceflight.

We therefore undertook a study to determine ASM levels in *Caenorhabditis elegans* (*C. elegans*), a small, yet complex organism with a neuromuscular system: (Szewczyk and Jacobson, 2005). *C. elegans* is a small nematode that is very similar genetically to humans (Altun and Hall, 2006). In fact, almost 40% of its genes are closely related to those of humans and *C. elegans* is recognized as a suitable model for studying human disease in space (Adenle et al., 2009). *C. elegans* has three ASMs, but this study focuses on ASM-1 and ASM-2. Both ASM-1 and ASM-2 are 30% homologous to the human ASM (Lin et al., 1998).

In this study, *C. elegans* were sent to the ISS to experience microgravity, while control *C. elegans* remained on Earth. We hypothesized that the worms on the ISS would develop muscle atrophy and experience increased ASM levels compared to the ground experiment. Atrophy extent was determined by morphometric analyses. ASM expression was determined by measuring protein levels. Overall, this study aimed to elucidate the role of ASM in muscle atrophy in microgravity, which may have additional implications for diseases characterized by muscle disuse and wasting.

**MATERIALS AND METHODS**

**Housing and Feeding of *C. elegans***

This experiment was conducted within a Type 3 Fluids Mixing Enclosure (FME) system (NanoRacks, Houston, TX, USA), designed for space research. FMEs have been used for all
previous SSEP experiments (Warren et al., 2013). As depicted in Figure 1, the FME is a flexible silicone tube, divided into three 2.8 mL compartments by plastic clamps. Sterilization of the FME minilab was achieved by autoclaving the tube at 121°C for 20 minutes, spraying the caps and clamps with 70% ethanol followed by exposure to ultraviolet (UV) light for 1 hour.

![Figure 1. Type 3 Fluids Mixing Enclosure (FME) Mini-Lab. The FME is 17 cm in length with a total volume of 8.4 mL divided into three 2.8 mL compartments. Volume 1 and 3 contained 2.4 mL of CeMM and Volume 2 contained 2.4 mL of CeMM housing 5,000 *C. elegans.*](image)

*C. elegans* maintenance medium (CeMM, a gift from Dr LR. Garcia, Howard Hughes Medical Ins, Texas A&M University) was used as the food source and growth medium in the FME. CeMM was used instead of *E. coli*, the traditional food source for *C. elegans* (Avery and You, 2012), because *E. coli* is viewed as a toxic risk on the ISS. 2.4 mL of CeMM was added to each of Chambers 1, 2, and 3 of the FME. In Chamber 2, 5,000 *C. elegans* were added to the 2.4 mL of CeMM. This compartmentalization ensured that the amount of food available to the worms could be controlled and rationed by unclamping and re-clamping the tube. The worms loaded were at mixed stages of development, which helped ensure their survival under the unpredictable conditions during pre-flight storage and shipment (Szewczyk, 2005). The FME systems were assembled at room temperature in the ground laboratory. They were sealed on both ends with plastic caps, twist ties, and gel, and were placed into a polyethylene bag to prevent contamination. The experimental FME, transported to the ISS by carriers organized by NASA, was unclamped twice by astronauts on the ISS: on the day of arrival on the ISS (day 25) and 14 days before the end of its ISS stay (day 50). A second, identical experiment was conducted on Earth as a control. Worms were harvested from both systems on day 70 after tube sealing. The space *C. elegans* spent a total of 40 days at microgravity. Table 1 shows a complete timeline of the experiment, including changes in location, temperature, and state of the FME during its round trip to the ISS and back.

Prior to the launch, we determined the optimal ratio of CeMM to air in each volume of the FME for maximum survival of *C. elegans*. We optimized our ratios by assembling FMEs containing variable volumes of CeMM and air and observed the worms to determine which conditions maximized survival. We observed the highest number of live *C elegans* after 70 days using 2.4 mL CeMM/compartment (2.1 worms/µL), compared to 2 mL CeMM/compartment (2.5 worms/µL), and 2.8 mL CeMM/compartment (1.8 worms/µL). Therefore, the optimal ratio of 2.4 mL of CeMM and 0.4 mL of air was selected, as this ensured that there were live nematodes in the FME throughout the ten-week experimental period.
Table 1. Timeline of the experiment. The letter A stands for arrival (at the ISS) and the letter U stands for undock from the ISS.

<table>
<thead>
<tr>
<th>Location</th>
<th>Time Start</th>
<th>Time Duration</th>
<th>Temperature (°C)</th>
<th>Changes made to the FME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Travel from Toronto to Houston (FedEx)</td>
<td>A-25 days</td>
<td>2 days</td>
<td>2-4</td>
<td>None</td>
</tr>
<tr>
<td>NanoRacks (Houston)</td>
<td>A-23 days</td>
<td>12 days</td>
<td>2-4</td>
<td>None</td>
</tr>
<tr>
<td>Travel from Ferry to Launch</td>
<td>A-11 days</td>
<td>11 days</td>
<td>2-4</td>
<td>None</td>
</tr>
<tr>
<td>Rocket travel to ISS</td>
<td>A-6 hours</td>
<td>6 hours</td>
<td>2-4</td>
<td>None</td>
</tr>
<tr>
<td>ISS</td>
<td>A</td>
<td>6 weeks</td>
<td>21-24</td>
<td>A+0 days: Unclamp Clamp A, shake gently for 3 seconds U-14 days: Unclamp Clamp B, shake gently for 3 seconds</td>
</tr>
<tr>
<td>Travel from Landing to Houston</td>
<td>U</td>
<td>4 days</td>
<td>0-10</td>
<td>None</td>
</tr>
<tr>
<td>Travel from Houston to Toronto (FedEx)</td>
<td>U+4 days</td>
<td>24 hours</td>
<td>2-4</td>
<td>None</td>
</tr>
</tbody>
</table>

**C. elegans Viability and Morphometric Analyses**

Following return from space, the number of *C. elegans* in both the experimental and ground FMEs were counted. A small equivalent aliquot of worms was taken from each tube, viewed with a phase contrast microscope, and photographed. The length and cross-sectional area of live *C. elegans* were measured, using ImageJ software, in 40 randomly chosen individual worms from each sample. The average length and area of the worms were then calculated for both the space and ground worms.

**SDS PAGE and ASM Immunoblotting**

To create the *C. elegans* protein lysate, both tubes of worms were washed three times with M9 solution, once with phosphate buffered saline (PBS), and then re-suspended in 75 μL of ice-cold lysis buffer (25 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA, 0.25% NP40, 1 mM PMSF, 1 mM Na₃VO₄, 2.5 mg/mL Pepstatin-A, 10 mM NaF, and 1 Protease Inhibitor Cocktail Tablet [Roche, Basel, Switzerland] per 10 mL solution). The samples were incubated on ice for 30 minutes, then subjected to three cycles of flash freezing in liquid nitrogen, partial thawing, and sonicaton for 5 seconds at 7W. Samples were then incubated at 4°C with agitation for 30 minutes and centrifuged at 13,000 rpm. Supernatant protein concentration was determined with the BCA assay (Thermo Scientific, Waltham MA, USA).

Worm lysate samples (30 μg) were separated by 10% SDS-PAGE, transferred to nitrocellulose, Ponceau-S stained, and immunoblotted with rabbit anti-acid sphingomyelinase antibody (sc-11352 Santa Cruz, CA, USA; 1:500 dilution). Prepared samples were analyzed with IRDye 800 CW fluorescent secondary antibody (Mandel Scientific, Guelph ON, Canada; 1:10,000 dilution). Quantification of the Li-Cortm fluorescent signal was performed with the LiCor Odyssey FC (Mandel Scientific). Western blotting
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for GAPDH (ab A245 Abcam, USA; 1:10,000 dilution detected with Mandel Scientific IRDye 680 RD, 1:10,000 dilution) was performed as a loading control. Samples were run in duplicate.

**Statistical Analysis**

Comparisons between the space worms and control worms were conducted using the Student's t-test. Statistical significance was determined if p<0.05.

**RESULTS**

***C. elegans Size and Morphometrics***

The space worms were received at the lab 70 days after initial loading into the FME. There were 72,050 live space worms, compared to 95,200 live ground worms. As illustrated in Figure 2, the space worms were significantly larger in length and in cross-sectional area than the ground worms. The mean length of the space worms was 0.42±0.13 mm, while the ground worms exhibited a mean length of 0.29±0.076 mm. The mean cross-sectional area of the space worms was 0.0065±0.0031 mm², while the mean cross-sectional area of the ground worms was only 0.0042±0.0021 mm². The histograms in Figure 3 demonstrate a broader range of distribution of length and cross sectional area for the space worms compared to the control ground worms.

![Figure 2](image.png)

*Figure 2.* Length and cross-sectional area (CSA) of ground and space worms. Representative images of (A) ground worms, and (B) space worms are shown. Live *C. elegans* appear curled and dead *C. elegans* assume a straight and thick shape. Only live *C. elegans* were measured. The space worms (N=40) were longer (C), and had larger CSA (D), compared to the control worms (N=40). Data are mean ± SD. ***p<0.05.
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Figure 3. Histograms of length and cross-sectional area (CSA) of space and control *C. elegans*. The top panels depict the distribution of lengths of randomly selected control *C. elegans* (n=40, left panel) and space *C. elegans* (n=40, right panel). Bottom panels similarly demonstrate the distribution of CSAs of control (left panel) and space (right panel) worms. A broader distribution of both length and CSA is evident in the space worms compared to the control ground worms.

ASM Levels

As illustrated in Figure 4, the space worms exhibited reduced ASM-1 and ASM-2 expression, compared to the ground worms.

The space worm lysate had a higher protein concentration, compared to the ground worm lysate, as determined by a BCA assay (Thermo Scientific). The space worm lysate had a protein concentration of 6.667 μg/mL, whereas the ground worm lysate had a protein concentration of 4.286 μg/mL.

DISCUSSION

This is, to our knowledge, the first study completed to assess ASM protein expression in *C. elegans* in microgravity. The purpose of the experiment was to gain a better understanding of the molecular regulation of muscle atrophy in microgravity, which may also provide insight into the mechanisms of atrophy in disease. We hypothesized that oxidative stress and altered ASM expression are involved in the induction of muscle atrophy that results from both sustained exposure to microgravity, as is seen in astronauts, and from ALS. We predicted that ASM levels would be elevated in *C. elegans* exposed to microgravity, as sphingomyelinase gene expression levels and ceramides have been found to increase in mouse models of ALS (Henriques et al., 2018; Cutler et al., 2002) We also aimed to test whether the nematodes’ muscle mass would be impacted by the microgravity conditions.

We found that *C. elegans* exposed to microgravity were longer, had greater cross-sectional areas, and had markedly reduced ASM-1 and ASM-2 expression compared to the control worms. These are striking and unanticipated results, contrary to our hypothesis and previous literature. Muscle atrophy is observed in humans in spaceflight and previous studies have shown...
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Figure 4. Ground and space worm lysates immunoblotted for ASM-1 and ASM-2. The space worms exhibited decreased expression of both ASM-1 and ASM-2, compared to the ground worms (left panel ASM Western blot, right panel graph of quantified fluorescent signal). GAPDH immunostaining demonstrates equivalent protein loading. Data are mean ± SD. ***p<0.05, **p<0.1.

Similarly, *C. elegans* in microgravity experience decreased rates of myogenic transcription and have lower muscle density (Higashibata et al., 2006). Higashibata (Higashibata et al., 2016) also reported a decrease in length and fat accumulation in the space flown *C. elegans*. Furthermore, ASM-2 gene expression analyses using cDNA microarrays from International *C. elegans* first experiment (ICE-FIRST) (Selch et al., 2008) and *C. elegans* RNAi space experiment (CERISE) (Higashitani et al., 2009) showed a 1.3 fold increase and a 1.1 fold decrease, respectively, although these results were not statistically significant (unpublished data, personal communication with Nathaniel J. Szewczyk).

Interestingly, Kim and Sun (2012) report that inactivation of the ASM homologs leads to slower reproduction and increased longevity of *C. elegans*. Given the lower expression levels of both ASM-1 and ASM-2 in the space worms, we speculate this may have increased their longevity, resulting in an increase in size compared to the control worms. Furthermore, slower reproduction meant that there were fewer worms in the space tube than in the control tube. As a result, each space worm had greater access to nutrients and oxygen, which might also explain why the space worms were able to grow larger. The reason for the decrease in ASM levels in space worms relative to the control worms is not known. ASM expression and activity is influenced by stress (Chung et al., 2016), and stressors may have impacted the two cohorts of worms differentially, but this was not assessed in our study.

Of interest, we were able to demonstrate that a population of *C. elegans* can survive for 10 weeks within the confines of a 17 cm FME; to our knowledge this is a novel finding. A previous study completed as part of the SSEP program did not yield live *C. elegans* upon return to Earth, due to an error in feeding the worms during spaceflight. This experiment concluded that the FME could not produce viable populations for post-flight analysis on extended missions such as the SSEP missions (Warren et al., 2013). With the return of 72,050 live *C. elegans* from the ISS, our study demonstrates that nematode populations can survive with limited oxygen and nutrients for at least 70 days. Not only did the worms survive space travel, but the population grew to more than fourteen times its original size. Therefore, *C. elegans* may be used as a model organism for...
future prolonged microgravity experiments in FME tubes.

There were a number of limitations to our study, as a result of regulations set by NanoRacks, NASA, and SpaceX. Only one tube could be sent to space, thereby permitting no experimental replicates, which limited the power of our experiment. Although we could not send multiple FME tubes to the ISS, we maximized the number of *C. elegans* within the tube to account for variability between worms. Additionally, the experiment needed to be performed in an FME, which was difficult to use. It was prone to leakage, contamination, and overall wear and tear. Although preliminary testing conducted by us to optimize experimental conditions allowed us to determine the most effective way to seal, subtle leakage or contamination during spaceflight could have impacted our results. In order to prevent leakage, two twist ties, gel, and a plastic cap were placed on each end of the tube; however, 1.5 mL of liquid was still lost due to leakage during the trip.

There were also several limitations of the experimental design. We did not test for the third ASM isoform, ASM-3, which may also affect the lifespan of the nematodes and their oxidative stress levels. We did not test for ASM-3 because a commercially produced antibody was not available. The anti-ASM antibody used was generated against the human ASM, and while it recognized proteins at the correct molecular weight of ASM-1 and ASM-2, it also recognized several other bands of unknown significance. These bands may have been due to non-specific reactivity or reactivity with degradation products. It is unclear why these bands were only seen in the ground control worms, given that GAPDH levels indicated equal loading of ground and space worm lysates. Furthermore, we did not evaluate oxidative stress, ASM-1 or 2 activity, or evaluate behavioral changes in the worms, which may be influenced by oxidative stress and could have served as a surrogate marker (Possik and Pause, 2015). Although this data may have enriched our knowledge, we were mainly focused on the impacts of microgravity on ASM expression. Lastly, *C. elegans* size is in part dependent on maturation stage. Notably we did not evaluate maturation stage of the space or ground worms, which may have confounded our morphometric measurements.

In summary, as high school students working within the SSEP program, we have importantly demonstrated that *C. elegans* can survive for at least 70 days with limited nutrients, space, and oxygen and are therefore a feasible and effective model for spaceflight and for SSEP experiments. We also demonstrated a statistically significant difference in expression of ASM between ground and space worms, although the underlying mechanisms are not known. Future research should aim to further evaluate the role of ASM in muscle atrophy in both spaceflight and in diseases such as ALS, as this may enable the identification of targets and strategies for therapeutic manipulation.

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Investigation of Zebrafish Larvae Behavior as Precursor for Suborbital Flights: Feasibility Study

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ABSTRACT

Suborbital spaceflights, carrying scientific payloads, allow scientists not only to test the feasibility of their payloads, but they also provide the basis for refining scientific hypotheses to be later tested on the International Space Station (ISS). Therefore, it is essential to establish robust pre-flight procedures in order to take advantage of this unique research platform to facilitate payload delivery. In the present study, we assessed zebrafish larvae behavior as a precursor for the future suborbital spaceflight involving research on the musculoskeletal system. Zebrafish larvae were exposed to the same physiological stressors they would encounter during suborbital spaceflight: alterations in light, thermal, and centrifugation conditions. Their behavioral responses were analyzed using the DanioVision (Noldus) behavioral tracking system. Our results showed that zebrafish were most active when kept in a dark environment as measured by swim distance. Also, thermal alterations revealed that zebrafish larvae adapted well to the different temperatures ranging from 25°C to 32°C with the highest levels of locomotor activity observed at 32°C. Finally, the centrifugation tests demonstrated that although zebrafish were exhausted initially, their recovery process was short, lasting for approximately five minutes. Taken together, our findings support the hypothesis that using zebrafish larvae is a feasible model for future suborbital flights. Thus, the lessons learned allow us to propel this research with more refined and realistic procedures as a precursor for orbital flights to the ISS and to cis-lunar space.

INTRODUCTION

Suborbital spaceflights are becoming an important scientific research platform offering a wide range of new research opportunities and enabling scientists to use microgravity as a unique environment to develop and refine hypotheses to be later tested in orbital space (Wagner et al., 2009; Moro-Aguilar, 2014; Pletser et al., 2016). Suborbital research is currently being undertaken with reusable vehicles such as Blue Origin’s New Shepard and Virgin Galactic’s SpaceShipTwo. We have flown a scientific payload onboard Blue Origin’s New Shepard as part of the Research
Education Mission (REM) M7 in December of 2017. Other emerging suborbital research platforms include the American Vector Space Systems’ Vector-R vehicle and New Zealand’s Electron launch vehicle. Additionally, Europe’s first reusable suborbital rocket, PLD Space’s Arion 1, plans to conduct test flights in 2019, with a focus on flying new research science and technology hardware while providing a wide breadth of educational applications for students to send suborbital payloads, such as micro/small satellites and NanoLabs. Given the rising interest in using suborbital spaceflights for scientific purposes, it is essential to test, validate, and optimize the procedures for scientific payload preparation and integration.

The Spaceflight Operations team in the Applied Aviation Sciences (AAS) department at the Embry Riddle Aeronautical University (ERAU) is currently developing several suborbital payloads as part of the Arete STEM (Science, Technology, Engineering, and Math) Project (ARETE) to demonstrate joint commercial spaceflight activities. The AAS department in the College of Aviation (COA) has been allotted a flight opportunity to send a scientific experiment to suborbital space onboard Blue Origin’s New Shepard capsule at the end of 2018, early 2019. ERAU is working together with the scientists from the University of Texas Health Science Center at San Antonio and the Medical University of South Carolina on the preparation of a translational science research project aimed at investigating the effect of microgravity on the musculoskeletal system.

It is well established that prolonged space travel has detrimental physiological effects on the human body, particularly the skeletal muscle. As previous studies indicate, the musculoskeletal system is impaired with prolonged duration of weightlessness (Fitts et al., 2000; Fitts et al., 2010; Trappe et al., 2009; Bagley et al., 2012). Long stays in microgravity also severely affect bone density deterioration (Hodkinson et al., 2017), such as bone losses in the spine, femur neck, trochanter, and pelvis. In addition, it is well reported that muscle atrophy is a significant adverse effect from space travel. Furthermore, despite exercising, muscle mass has been shown to decrease dramatically during long duration spaceflights (Gopalakrishnan et al., 2010; LeBlanc et al., 2000). According to NASA, astronauts experience up to a 20 percent loss of muscle mass on spaceflights lasting 5 to 11 days (NASA, n.d.). Moreover, muscle atrophy is also considered a major symptom of many patients with mitochondrial disease, which affects the energy production required for their muscle integrity (DiMauro, 2004). Unfortunately, there is no definitive treatment nor is there a cure for muscle atrophy. Given the upcoming projected long term space travel missions to the Moon, Mars, and beyond, it is essential to use state-of-the-art scientific models to further investigate the impact of microgravity on the musculoskeletal system as well as to identify preventive measures mitigating the severe consequences of extended exposure to weightlessness. In fact, the Space Biology Plan for 2016-2025 released by NASA encourages studies designed to investigate the biology effects of long duration space environment exposure (Tomko et al., 2016).

In this study, we assessed the behavior of zebrafish larvae as a precursor for the future suborbital and orbital flights that will be aimed at investigating the role of space-induced muscle atrophy. To determine the extent to which zebrafish larvae could survive the suborbital flight, we exposed them to the same stressors they would encounter during the actual suborbital flight: alterations in light, thermal, and vibration conditions. Zebrafish make an excellent research model and have many advantages, such as their transparency, fast development, and genetic similarity to humans (Tavares and Santos Lopes, 2013; Chan et al., 2018; Aceto et al., 2016). Furthermore, a recent review suggests that zebrafish mutants and transgenic lines can be used to model human skeletal diseases (Laizé et al., 2014). Specifically, it applies to studying the osteocytic bone, multinucleated osteoclasts, collagen in bone, and various types of cartilages and ossification of the vertebral column with the hope to better understand the underlying mechanisms of diverse musculoskeletal disorders. In addition to using zebrafish as a research model, some researchers (Chatani et al., 2015; Chatani et al., 2016; Ijiri, 1995) have used medaka fish to study microgravity induced effects. These studies revealed that medaka fish exposure to microgravity resulted in impaired physiological function with a change in mechanical force.
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(Chatani et al., 2015) and altered gene expression in osteoblasts and osteoclasts (Chatani et al., 2016).

Zebrafish behavior is a direct reflection of neural activity and its modulation by external stimuli (Girdhar et al., 2015; McKeown et al., 2009). Today’s technology allows the use of high-throughput automated zebrafish tracking systems to generate quantitative results to capture their behavioral responses as readouts of their wellbeing (Ingebretson and Masino, 2013; Liu et al., 2015; Zhou et al., 2014).

MATERIALS AND METHODS
Zebrafish Larvae

Wild-type AB strain zebrafish were crossed according to standard methods and embryos were raised to 6-8 days post-fertilization (dpf) in accordance with Westerfield (2007). Zebrafish were maintained in petri dishes (100 mm diameter) filled with embryo water in a 28.5°C incubator under a 14/10 h light/dark cycle. Zebrafish were exposed to three types of stressors: alterations in light, thermal, and centrifugation conditions. Their locomotor response to these stressors were tracked using the DanioVision® instrument (Noldus) and subsequently assessed using EthoVision® software (Noldus) (Rahn et al., 2014). The behavioral recordings took place by transferring larvae from petri dishes into individual wells of a 48-well plate using 1 mL pipette with a cut tip containing 500 μL of water. This procedure was repeated for each batch of zebrafish that was exposed to a different stressor. Once zebrafish were placed in the 48-well plate, we proceeded to calibrate the DanioVision instrument to track their motion. Given swimming performance is a biological characteristic that has a very important role on fish survival, our main readout was the movement of the zebrafish, also referred to as distance traveled. All animal studies were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (#180278) and performed in accordance with the guidelines.

Calibration of the DanioVision® Instrument

The calibration process required input information pertinent to our experiment, such as live tracking on subjects (in this case zebrafish larvae) and the arena template: 48 round-well plate at 30 frames per second. Once the reference plate (empty plate) was placed into the instrument, we set the dynamics subtraction and the acquisition settings (e.g., distance traveled). After the instrument was properly calibrated with the empty plate, we proceeded to place the plate containing zebrafish and set up the light conditions using the EthoVision® software. This calibration process was critical for obtaining optimal tracking results, and for maximizing the number of data points tracked by the instrument.

Experimental Procedures
Light condition procedures

To assess the effect of light on zebrafish movement, twenty one zebrafish (6 dpf) were transferred into individual wells in a 48-well plate. In general, zebrafish show good spontaneous motion between six days and four weeks post-fertilization. Thus, better tracking is obtained as the zebrafish gets older. As depicted in Figure 1, we conducted a study where we first placed zebrafish in wells C2 to C6 and D2 to D6. Other zebrafish were placed in wells B2 to B7 and E2 to E6, while the A wells were left empty. To set up this test, we placed the 48-well plate with the zebrafish into the calibrated instrument with a 30 min delay to acclimate the fish to the current condition. The instrument started tracking for the next 10 min, which is a typical timeframe for a suborbital flight on Blue Origin’s New Shepard. Video was taken over a 10 min period starting with 5 min of lighted conditions followed by 5 min of dark condition. A switch condition was applied in between lighted and dark conditions. Locomotor activity was measured for 4 min of the lighted portion and for 5 min of the dark condition as displayed in Figures 3-6.

Thermal procedures

Ten zebrafish larvae (8 dpf) were placed in 15 mL conical tubes containing 14 mL of water and were exposed to the following temperatures: 25°C, 28.5°C (reference temperature), and 32°C for 2 days. Thermal stress was performed at 3.5°C above and below the reference temperature. Afterwards, larvae were transferred into the 48-well plate using the following template: rows A and B had no zebrafish, row C contained larvae...
exposed to 32°C, while rows D and E contained zebrafish exposed to 25°C and 28.5°C, respectively.

Centrifugation procedures

For the centrifugation test, zebrafish larvae (n=10) were placed in 15 mL conical tubes containing 14 mL of water and were centrifuged for 5 min at 10 g. Centrifugation was performed at 28.5°C, since this is the optimum temperature for zebrafish. Zebrafish exposed to 25°C underwent centrifugation stress, while zebrafish exposed to 32°C were not exposed to centrifugation stress, as zebrafish exposed to temperatures above the reference temperature (control) are more sensitive than those exposed to temperatures below. Previous studies (Scott and Johnston, 2012) support the fact that high cruising speed zebrafish are observed at warmer temperatures. Therefore, we decided to not put additional stress on the zebrafish. However, this will be addressed in future studies. After centrifugation, zebrafish larvae were transferred into a 48-well plate using the following template: wells A1 to B1 had no zebrafish, B2 to C5 contained zebrafish that did not undergo centrifugation, while wells C6 to D3 contained zebrafish that were exposed to centrifugation.

Tracking

After exposing zebrafish to the above conditions, the locomotor activity of the zebrafish was recorded for 11 min (660 s), the same as the total time of a suborbital mission. The motion was sampled at 24.55 Hz generating 16204 positions (X, Y) and velocity data samples per individual. The tracking software provided information at the end of the simulation of missed samples and samples not found (expressed as a percentage). If the fish were not found, then the path of the zebrafish would appear in yellow instead of blue (zebrafish was tracked). In our thermal test, the instrument tracker only missed 0.1% of all the samples obtained in all the wells where the zebrafish were located and 0.1%-0.2% of all the samples. Thus, the overall tracking performance of the instrument was excellent, as the zebrafish tracking efficiency was about 99.8%.

Data Extraction and Analysis

The locomotor activity data for the zebrafish was obtained using the EthoVision XT® software. Each observation was stored in an Excel data file containing the recording time in seconds, and the X center and Y center coordinates of the zebrafish larvae. The motion of the zebrafish was captured by an IR sensor that would take 30 frames of the zebrafish every second in a 48 round-well plate. The sensor detected the 2-dimensional position (X, Y) and velocity of the zebrafish during each frame but did not provide the Z position of the zebrafish. We approximated the Z position as follows: we added a 500 μL volumetric solution pipetted into each of the 13 mm diameter wells. Thus, the height of the cylinder where the zebrafish were swimming was about 1-1.28 cm. We assumed the zebrafish swam within this volume.

We have approximated the turning speed, \( \omega \), also known as the angular velocity, of each fish at each step as follows (Zienkiewicz et al., 2015):

\[
\omega = \text{sgn}[\vec{v}(t + \Delta t) \times \vec{v}(t)] \cdot \frac{1}{\Delta t} \cos^{-1}\left(\frac{\vec{v}(t + \Delta t) \cdot \vec{v}(t)}{||\vec{v}(t + \Delta t)|| \||\vec{v}(t)||}\right)
\]

where \( [\cdot]_z \) indicates that the sign of the z-component of the cross product provides you the turning direction and a positive sign denotes movement in the anti-clockwise direction. The change in time, \( \Delta t \), is defined as the inverse of the sampling frequency (\( f_s=30 \) Hz). This turning speed of the zebrafish is associated with the rate of the change of the orientation of the velocity vector. Our model also computes the radial and tangential velocity of the zebrafish at each time step. In our results section, we will show how the turning speed or angular velocity parameter can be expressed in contour maps having the tangential velocity in the Y-axis and the radial velocity in the X-axis in order to have a better understanding of the swimming behavior of the zebrafish.
RESULTS

Light Conditions Study

In this study phase, we exposed zebrafish to different light conditions for various time durations. First, the zebrafish were exposed for 5 min in the lighted condition (100%), then the light was turned off for 6 min (Figure 1). Data was analyzed during the first 5 min under lighted conditions, 1 min after the light was turned off, and in the last 5 min of dark conditions. Total amount of this exposure (10 min) mimics the duration of the suborbital spaceflight.

During the first 5 min under lighted conditions, the average distance that zebrafish larvae moved from the center-point for all wells was 91.26 mm (9.13 cm). The maximum distance traveled measured was 173.76 mm (17.4 cm) observed in C6 (well number 22 in Figure 1), while the minimum distance was 9.21 mm (approximately 1 cm), observed in C2 (well number 18 in Figure 1). We used the following notation (Figure 1): well numbers 2-8 will be indicated by A; well numbers 9-16 by B; well numbers 17-24 by C; well numbers 25-32 by D; well numbers 33-40 by E; and well numbers 41-48 by F.

The average distances moved in the B2-B7 (red), C2-C6 (black), D2-D6 (blue), E2, E3, E5-E6 (magenta), and F4, F6 (green) wells were 9.37 cm, 11.53 cm, 10.23 cm, 7.61 cm, and 1.43 cm, respectively. Second, we turned off the light to measure the startle response to the change in light condition in the next 1 min. The distances moved in the same wells by the zebrafish larvae were 6.23 cm, 7.80 cm, 12.03 cm, 8.16 cm, and 3.06 cm, respectively. The average traveled distance was 74.56 cm with a maximum distance traveled of 18.98 cm observed in E5, and a minimum distance traveled of 0.08 cm in B6. Third, we kept the light off (100%) during the last 5 min and...
observed high activity of the zebrafish larvae as seen at the bottom of Figure 1. The average distance moved by the zebrafish in the wells was 23.24 cm, 30.04 cm, 23.64 cm, 20.82 cm, and 8.77 cm, respectively. The maximum distance traveled was 51.79 cm observed in well C5, and the minimum distance traveled was 1.61 cm in well F6.

In Figure 2, we compared the distance traveled (in mm) during the first 5 min period when the light was on (dashed lines) and during the last 5 min period when the light was off (solid lines). Our results indicate that zebrafish were more dynamic when the light was off. The maximum distance moved by zebrafish was about three times higher for dark conditions as compared to the light conditions.

![Distance Traveled Comparison](image)

**Figure 2.** Comparison of total distance traveled of zebrafish larvae during various light phases. 5 min light on is represented by the dashed line and 5 min light off is represented by the solid line. Color representations are as in Figure 1.

To further demonstrate the effect of light on zebrafish movement, we analyzed zebrafish behavior derived from the individualized wells of the 48-well plate (Figures 3-6). Our data demonstrate that in B wells zebrafish are the most active when exposed to the dark environment. This finding was consistent throughout the rest of the wells with a few exceptions observed in wells E5 and C2 where the difference in distance traveled was not significant between light and dark conditions.

Statistical evaluation in Figures 3-6 for the average motion was analyzed for each of the three conditions: 4 min with light on, 1 min switch condition, and 5 min light off at each of the wells B, E, C, and D (Figure 1). This was the order chosen so that control zebrafish were in B wells, zebrafish at 28.5°C were in E wells, zebrafish at 32°C were in C wells, and zebrafish at 25°C were in E wells.

Wells B2-B7 showed an average motion during each of these conditions of 31.15%,
26.73%, and 78.23%, respectively. Wells E2-E7 (zebrafish in E4 well was dead) corresponded to an average motion of 50.73%, 40.82%, and 75.18%, respectively. Wells C2-C6 displayed an average motion of 48.78%, 51.68%, and 88.20%, respectively. Well D2-D6 had zebrafish with an average motion of 51.94%, 40.00%, and 81.14%, respectively. Taking the mean of the means for each condition, we observed that the average motions across all the wells for each of the three conditions were 45.90%, 39.81%, and 80.69%, respectively. This statistical analysis showed that zebrafish were about 1.8 times more active during the 5 min light off condition than during the 4 min light on condition, and 2.0 times more active than during the switch condition.

**Figure 3.** Behavioral motion of zebrafish larvae in B wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.
We also studied the average motion of zebrafish between each condition for each well. For B wells (control), the zebrafish average motion increased about 7.6% from the condition at 4 min with light on to the 1 min switch condition, and the average motion increased 38.5% from the switch condition to the condition at 5 min with light off. For E wells, the average motion was decreased about 9.9% and increased 26.2% between conditions, respectively. For C wells, the average motions between conditions increased by 2.9% and by 36.5%, respectively. Finally, for D wells the average motions between conditions were 4% decrease and 39.1% increase, respectively. These results show an increase of 35.1% in the average motion going from the 1 min switch condition to the 5 min with light off condition.

Figure 4. Behavioral motion of zebrafish larvae in E wells during different light conditions. The blue dots represent the distance traveled by larvae during the 5 min lighted condition. The red crosses denote the distance traveled by larvae during the switch condition. The black crosses correspond to the distance traveled by larvae under the 5 min dark condition.
Thermal Conditions Study

Next, we conducted a feasibility study of the zebrafish larvae under various temperatures: 28.5°C (reference temperature), 32°C, and 25°C. This zebrafish testing profile was based on a more realistic light conditions scenario based on Blue Origin’s New Shepard Crew Capsule flight profile, which lasts about 10 min. Thus, we initially exposed the zebrafish larvae during 3 min of light conditions (mimicking ascent), followed by 1 min transition from dark to light, followed by 3 min in dark light conditions (mimicking darkness in microgravity), followed by 1 min transition from dark to light, followed by 3 min in light conditions (mimicking descent).

Figure 7 displays the trajectory of these zebrafish larvae when exposed during the above
sequence of light conditions at various temperatures. It was observed that the larvae followed more defined circular paths when exposed to 32°C as seen in the two top plots of Figure 7. The larvae followed a similar circular path, although not as well-defined, when exposed to the reference temperature of 28.5°C with various passages through the center of the well as shown in the center two pictures of Figure 7. These zebrafish larvae showed more irregular paths when exposed to 25°C as observed in the bottom two plots of Figure 7. Because zebrafish larvae locomotor activity is affected by temperature variations, an active thermal system may be required to have a sustainable life support system to increase the life of these zebrafish larvae to ensure survivability, especially if the launch is slipped or scrubbed as occurred with the
Blue Origin launch on December 12, 2017. Our experience during the last Blue Origin New Shepard M7 revealed drastic temperature variations during the pre-flight, in-flight, and post-flight operations. The payload, which is integrated at the Payload Processing Facility (PPF) at the West Texas Launch Site (WTLS), must be handed off to NanoRacks and Blue Origin teams before integration into the New Shepard’s Crew Capsule at least 7 hours before the launch. Because of this, standard care of these larvae will require an appropriate life support system, proper handling, care, and pain mitigation protocols in order to be airworthy for the suborbital launch.

Interestingly, the zebrafish larvae activity displayed in C4 and C8 wells that were exposed to 32°C had the highest distance traveled and the highest mean velocity (Figure 7). Specifically, zebrafish larvae in C8 well moved a total distance of 78.46 cm over the time period of about 10 min with a mean velocity from the center point of 1.46 mm/s, while larvae in C4 well moved a total distance of 68.04 cm with a mean velocity of 1.26 mm/s. Larvae activity depicted in E1 and E5 wells (exposed to 28.5°C) moved a total distance of 44.81 cm and 35.75 cm with mean velocities of 0.83 mm/s and 0.66 mm/s, respectively. Finally, larvae activity shown in wells D3 and D6 (exposed to 25°C) traveled total distances of 27.42 cm and 34.29 cm with mean velocities of 0.51 mm/s and 0.64 mm/s, respectively. Next, we also compared the locomotor activity of total distance for each configuration when we combined two stressors: temperature and centrifugation. Wells C1-C8 (only showing C4 and C8 wells) correspond to larvae exposed at 32°C with no centrifugation; wells D1-D8 correspond to larvae exposed at 25°C (centrifugation); wells E1-E7 correspond to larvae exposed at 28.5°C (centrifugation); and wells B1-B8 correspond to control larvae (no centrifugation). Control larvae moved an average total distance of 48.19 cm, larvae exposed at 32°C move an average total distance of 36.43 cm, larvae exposed to the reference temperature moved an average of 63.80 cm, and larvae exposed to 25°C moved an average distance of 37.82 cm.

Figure 8 depicts the distance traveled by zebrafish in their respective wells when exposed to various subsequent stressors. First, the Danio rerio were exposed to various temperature changes (25°C is shown in red, 28.5°C is shown in black, 32°C is shown in green, and control is represented by blue). After completion of this test, they were placed in the 48 well plate and exposed under different light conditions similar to what they would experience during about 10 min suborbital flight: 3 min in light condition representing the part of the ascent, 1 min going from light to dark condition, 3 min in dark representing suborbital altitudes, 1 min transitioning from dark to light, and 3 min in light condition again representing descent. Figure 8A shows the effect of centrifugation on 20 larvae. 80% of these showed distance displacements less than 6 cm and 20% of these showed distances traveled between 8 cm and nearly 13 cm. This indicates high fatigue levels on the zebrafish larvae. In the same graph we also observe that larvae traveled longer average distances when exposed to temperature of 32°C than in any other case. This observation is also noticed in the other plots in Figure 8. Larvae exposed to various thermal changes traveled longer distances than when exposed to centrifugation which confirms our observation of zebrafish being less active when they undergo spinning effects.

In general, Figure 8B to Figure 8E show that the larvae traveled longer distances at warmer temperatures (32°C) than at colder temperatures (25°C) with respect to the reference temperature (28.5°C), as indicated by the green and red lines, respectively. Another important observation in Figure 8 is that zebrafish larvae exposed to dark conditions traveled much longer distances than when they were exposed to light conditions. For example Figure 8C shows that 88% of all the larvae traveled distance larger than 10 cm and up to 50 cm is some cases while only 12% traveled less than 10 cm. If we compare this with Figure 8A, we observe that about 66% of larvae traveled less than 10 cm, and 44% traveled between 10 cm and 50 cm. Note that these larvae were exposed to 3 min light conditions at the start and end of the light cycle, as indicated in Figure 8A and Figure 8E, respectively. About 90% of these larvae traveled shorter distances during the last 3 min light cycle than during the first 3 min light cycle.
Figure 7. Behavioral motion of zebrafish larvae exposed to different temperature variations: 32°C (top), 28.5°C (middle), 25°C (bottom). Each red dot represents the position for each discrete data point tracked by Ethovision®. The blue line indicates the approximated path of the zebrafish between data points.
Figure 8. Distance traveled by zebrafish larvae when exposed to an 11 min varying light cycle. (A) 3 min in light. (B) 1 min light to dark. (C) 3 min in dark. (D) 1 min dark to light. (E) 3 min in light.
Centrifugation Study

In our final test, we performed the centrifugation phase as a final test in our study. Figure 9 depicts the centrifugation profile: maximum G-sensed acceleration in New Shepard is 4.7 g (red dashed line) for a few seconds during reentry (Blue Origin, 2017). Our study showed that zebrafish underwent angular velocities up to 300 rpm for 2 min, with a safety margin of about 2.3 or about 11 g. It is important to allow for a safety margin as an effective test to diagnose any forms of dysfunctional zebrafish activity that could be exacerbated by unexpected flight forces or flight anomalies.
This research will be further expanded later with various organisms so we could fly them on other national and international research platforms besides the Blue Origin’s New Shepard vehicle. In our future work, we will examine the effects of various hypergravity levels on these organisms (i.e., zebrafish) using various known simulation techniques, such as clinostats and rotating wall vessels, to better assess the performance of these organisms in space. As an example, previous research (Remus and Wiens, 2008) has examined the effects of hypergravity level on xenopus embryos that underwent centrifugation levels from $7\,g$ to $10\,g$.

Post-swimming recovery of zebrafish took about 3-5 min. Their physiology was notably impacted with most zebrafish being upside down in the conical tube as illustrated in Figure 10 (right). Very few zebrafish (3-4 zebrafish out of 10) were observed to have aberrant swimming activity after centrifugation stress since most of them were at the bottom of the tube as shown in Figure 10 (left). In addition, zebrafish showed very slow responses when they were tapped at the tip of the tube indicating they had a reduced startle response, while the more active zebrafish showed a higher response to these stimuli. Some zebrafish swam at an angle (tilting swimming),
exhibiting disorientation signs as shown by their droopy tail (right Figure 10). This lasted for about 4 min. All zebrafish were active again after 5 min. It is important to note that these effects were induced by the centrifugation process and not by using any of the neuroactive or neurotoxic substances that commonly provoke this type of behavior (Weichert et al., 2017; Lee and Freeman, 2014).

Furthermore, as illustrated in Figure 11, the exposure of zebrafish larvae to centrifugation revealed that this stressor led to the formation of more chaotic patterns of zebrafish movement as compared to the well-defined trajectories of fish not exposed to centrifugation as observed during 30 min data collection process with the DanioVision® (Noldus) behavioral tracking system.

Radial and Tangential Angular Velocity Maps

Figure 12 depicts various contour map representations of the radial and tangential velocities in mm/s for representative wells for a given condition. Figure 12A and Figure 12B show the radial and tangential velocities for wells C4 and C8, respectively. The maximum radial velocities were 80.58 mm/s and 87.28 mm/s, and the maximum tangential velocities recorded were 77.99 mm/s and 118.4 mm/s, respectively for these wells. Similarly, for wells E1 and E5 (Figure 12C and Figure 12D) the radial velocities were 105.6 mm/s and 43.2 mm/s, while the tangential velocities were 156.3 mm/s and 49.85 mm/s. The radial velocities for D3 and D6 wells (Figure 12E and Figure 12F) were 56.29 mm/s and 43.2 mm/s, and the tangential velocities were 35.38 mm/s and 44.91 mm/s, respectively. The means of the radial velocities for each well (C4, C8, E1, E5, D3, and D6) were 0.86 mm/s, 1.81 mm/s, 0.52 mm/s, 0.46 mm/s, 0.34 mm/s, and 0.44 mm/s, respectively. The means of the tangential velocities for these wells were 3.63 mm/s, 30.54 mm/s, 3.43 mm/s, 3.30 mm/s, 3.27 mm/s, and 3.30 mm/s, respectively. Note that both radial and tangential velocities are notably smaller for the larvae exposed to 25°C if compared with those velocities for larvae exposed at reference or higher than reference temperatures. The sum of all the radial and tangential velocities for each condition was analyzed. For instance, the sum of the radial velocities for larvae exposed to 32°C is greater than that for larvae at reference temperature and for larvae at 25°C. A smaller difference is observed between 32°C and 28.5°C conditions, which may explain better adaptability of the larvae at slightly larger temperatures than at lower temperatures. This behavior is consistent for other wells.

DISCUSSION

There is a significant interest in using ISS for both conducting research and habitation for the next decades. However, such missions are currently burdened by the severe consequences space has on human health. Therefore, there is a great need for effective multidisciplinary studies comprised of both basic and applied science aimed at producing effective countermeasures against the deleterious influences of spaceflight on the human body (Alwood et al., 2017).

In this study, we have shown that exposing zebrafish larvae to the same physiological stressors they would encounter during the actual suborbital flight leads to alteration of their behavior, but does not affect their survival. Furthermore, this study provided an insightful contribution to various stressor-based effects on the zebrafish larvae to establish a risk assessment of the model organism Danio rerio when designing future suborbital and orbital spaceflights. Our study is currently being extended by using clinostats as a ground lab research platform that will give us further understanding on the behavior of these organisms under different microgravity levels.

Although zebrafish is a diurnal animal that is active during the light phase of the light-dark circadian cycle (Facciol et al., 2017), our light study indicated that zebrafish were more active in the dark. Some literature suggests (Serra et al., 1999; Maximino et al., 2010) that zebrafish have a natural preference for a dark environment. However, there is a contradiction to this observation in the field as some studies report zebrafish preference for brighter light environments (Champagne et al., 2010; Gerlai et al., 2000). Despite the observed differences among investigators, it was important to us to confirm that zebrafish are capable of adapting to alterations in light conditions and also to determine light preferences so that proper future housing cubes can be made.
Figure 12. Contour maps for radial and tangential velocities at various temperatures in different wells: (A) Well C4 at 32ºC. (B) Well C8 at 32ºC. (C) Well E1 at 28.5 ºC. (D) Well E5 at 28.5ºC. (E) Well D3 at 25ºC. (F) Well D6 at 25ºC
It is well established that water temperature affects the swimming performance of the fish. Our thermal study revealed the differences in zebrafish movement patterns when they were exposed to various temperatures at various time durations. More specifically, zebrafish exposed to 32°C formed well-defined trajectories as well as were the most active as compared to those exposed to 28.5°C and 25°C. Our finding is consistent with the literature suggesting that zebrafish exhibit the highest swimming capacity when they are raised at 31°C, as compared to the worst performance at 22°C, and moderate activity at 28°C (Sfakianakis et al., 2011).

Next, we assessed zebrafish behavior after administering centrifugation test, the critical phase of the suborbital spaceflight. Our results demonstrate that zebrafish are exhausted initially as observed by their upside down swimming. Our observation of zebrafish avoiding swimming into certain areas could be explained by the gravitaxis (bottom dwelling and diving to the “safer” lower regions) as an indicator of physiological reaction to stress (Blaser et al., 2010; Stewart et al., 2010). Importantly, the recovery processes took only approximately 5 min until larvae were active again. Previous studies (Ijiri, 1995) on medaka fish showed that exposure to microgravity for about 15 days made the fish forget how to swim under normal gravity conditions on Earth, and the fish took 3 days to readapt. During this study, the fish were studied by relying on visual rather than on vestibular cues without being forced to swim aberrantly due to microgravity.

Finally, we computed the radial and tangential velocities of zebrafish exposed to various thermal phases. Our data suggests that lower radial and tangential velocities were observed for zebrafish exposed at 25°C as compared to higher temperatures with a slight difference between 32°C and 28.5°C conditions. Based on our previous observation of zebrafish being more active at higher temperatures, they tend to move in more defined and predictable patterns with higher tangential velocities.

Given zebrafish are very sensitive to various stressors, our team is currently designing a life support system that will be tested and integrated in our next suborbital payload. Some of the hardware will include state-of-the-art sensors to measure the environmental conditions and a microcontroller to regulate our desired conditions. The technology development will be discussed in a subsequent manuscript.

Altogether, our study confirms the likelihood of zebrafish larvae surviving the suborbital flight. Subsequent research efforts will be devoted to further investigate the responses of zebrafish in various microgravity environments (e.g., Moon, Mars, ISS), and from hypergravity to these microgravity levels using various simulation systems (Van Loon, 2016). Our long term goal is to continue research efforts in achieving advancements in human health exposed to space. Given the similarity of the zebrafish genome to humans, our goal is to use zebrafish larvae as a stepping stone for our future suborbital space experiments. Results from our studies and similar studies focusing on space-induced alterations on muscle atrophy could identify the mechanisms mediating these changes, which in turn could lead to the synthesis of new drugs or treatments benefiting not only the space travelers, but also patients on Earth with musculoskeletal disorders.

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Chronic Exposure to Altered Gravity During the Pregnancy-to-Lactation Transition Affects Abundance of Cytoskeletal Proteins in the Rat Mammary Gland

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ABSTRACT

The mammogenic, lactogenic, and lactopoetic effects of prolactin (PRL) in the mammary gland are mediated through a specific cytokine receptor, the PRL-receptor (PRLR). PRLR is anchored to the cytoskeleton and its activation, and subsequent signal transduction, is dependent on an integral/intact cytoskeletal organization. Previous studies revealed a down-regulation of PRLR and reduced metabolic output in the mammary gland of rats exposed to hypergravity (HG). Therefore, the objective of this study was to use quantitative immunohistochemistry to determine the effects of HG exposure during pregnancy on the pre- and postpartum abundance of the cytoskeletal proteins in the rat mammary gland. Pregnant rats were exposed to either 2xg [HG] or 1xg [Stationary control (SC)] from days 11 to 20 of gestation (G20) through postpartum days 1 (P1) and 3 (P3). Spectral characterization and quantitation of each antigen (actin, tubulin, cytokeratin, and vimentin) per lobule (n=3-7 lobules/micrograph; 4 micrographs/slide) was computed using the CRi Nuance multispectral system. At G20 and P3, increased (p<0.001) amounts of actin, tubulin, cytokeratin, and vimentin were detected in HG rats. Tubulin, cytokeratin, and vimentin were overexpressed (p<0.01) in HG group compared to SC at P1. These results suggest that atypical composition of cytoskeletal proteins contribute to the aberrant lactogenic signal transduction and associated reduced postpartum mammary metabolic output in rats exposed to altered inertial environment.

ABBREVIATIONS

G9 Gestation day 9
G20 Gestation day 20
HG Hypergravity
IHC Immunohistochemistry
P1 Postpartum day 1
P3 Postpartum day 3
PRL Prolactin
PRLR Prolactin Receptor
Px Pixel
SC Stationary Control
SEM Standard Error of the Mean
INTRODUCTION

The mammary gland undergoes significant structural and functional transformation during the physiological phases of pregnancy, lactation, and involution. These changes are evident in both the secretory (epithelial) and non-secretory (stromal) support structures of the organ. The epithelial alveolar and myo-epithelial cells facilitate synthesis and delivery of milk (Richert et al., 2000), while the non-secretory assemblage of the extracellular matrix provides key structural and metabolic support to the gland. The dynamic rearrangement of the extracellular matrix throughout the distinct developmental stages is vital to the biochemical and biophysical morphogenesis of the organ (Masso-Welch et al., 2000). Importantly, critical changes in the mammary gland’s parenchymal architecture, cytoskeletal remodeling, and acquisition of biosynthetic competence occur mainly during the postnatal period, primarily during pregnancy.

Hormonal signals are indispensable for orchestrating mammary gland development during mammogenesis, lactogenesis, and galactopoiesis. During pregnancy, the dramatic growth and functional differentiation of the gland is dependent on prolactin (PRL) secreted by the pituitary gland (Ormandy et al., 1997; Topper and Freeman, 1980). In addition, the role of PRL in alveologenesis, milk synthesis, and galactopoiesis in various species is well documented (Brisken et al., 1999; Hennighausen and Robinson, 2005). PRL also regulates the synthesis and transport of the complex mixture of nutrients dispersed in milk (Bole-Feysot et al., 1998; Ormandy et al., 1997). The distinct effects of PRL in the mammary gland are mediated principally by membrane-anchored receptors (Bole-Feysot et al., 1998). Notably, an intact cytoskeleton is critical for the transmembrane activation and transduction of these pleiotropic PRL signals through the prolactin receptor (PRLR) (Zoubiane et al., 2004).

The cytoskeleton is an interconnected lattice of microfilaments, intermediate filaments, and microtubules that extends throughout the cytoplasm. Its dynamic and adaptive configuration is vital for cell architecture, organelle movement, cytokinesis, and extracellular environment sensing (Flusberg et al., 2001; Hughes-Fulford, 2003). The cytoskeleton plays a key role in coordinating the communication between the extra- and intracellular signals in tandem with mediating cell-to-cell interactions (Janmey, 1998). Actin and tubulin are crucial for the PRL-dependent development of the mammary gland (Zoubiane et al., 2004). Aberrations in the cytoskeletal structure are known to disrupt intracellular signaling machinery and impact functional output (Flusberg et al., 2001; Hughes-Fulford, 2003). Therefore, precise cytoskeletal composition and assembly are instrumental in the global development of the mammary gland, architectural alignment of the mammary tissue elements, and synchronization of functionality through coordinated extra- and intracellular interactions (Xu et al., 2009).

Spaceflight and other gravitational load (g-load) studies have shown the diverse effects of altered gravity on reproduction and mammary gland function (Bojados and Jamon, 2011; Casey et al., 2015; Casey et al., 2012; Lintault et al., 2007; Megory and Oyama, 1984; Plaut et al., 1999; Simeoni et al., 2005). Interestingly, results from microgravity and hypergravity (HG) studies show a continuum of responses for a number of biological systems, including the mammary gland (Casey et al., 2015; Plaut et al., 2003). Although HG exposure had no effect on the number of pups born, 50% of the pups from HG dams do not survive beyond 48 h (Casey et al., 2012; Lintault et al., 2007). Furthermore, lipogenesis in the mammary gland is impaired in altered gravity (Casey et al., 2012; Lintault et al., 2007; Patel et al., 2008). Results from an earlier study suggest that HG-exposed mammary gland metabolic output may be mediated by factors other than PRL and glucocorticoid (Patel et al., 2008). Plausibly, exposure to HG alters the mammary gland microenvironment, which adversely impacts physiological output and potentially compromises pups’ survival.

The cytoskeleton is responsible for sensing changes in g-load and mediating structural remodeling and related signal transduction (Dai et al., 2006). Studies utilizing normal and cancerous human cells (Carlsson et al., 2003; Carmeliet et al., 1998; Rosner et al., 2006; Sciola et al., 1999), as well as plants (Braun et al., 2002; Himmelspach et al., 1999), have shown that exposure to diverse g-loads leads to an alteration in cytoskeletal organization and distribution.
Specifically, atypical levels of microfilaments, microtubules, and intermediate filaments have been associated with abnormal karyokinesis and cytokinesis (Carlsson et al., 2003; Carmeliet et al., 1998; Meloni et al., 2006; Rosner et al., 2006; Sciola et al., 1999). Although the above studies provide an understanding of the multiple effects of altered gravity on the mechanical support structures of a cell, there is no report on the effect of altered gravity on these proteins in a dynamic organ like the mammary gland across different physiological states.

Despite the description of the effects of HG on pregnancy and lactation (Casey et al., 2012; Lintault et al., 2007; Megory and Oyama, 1984; Plaut et al., 1999; Simeoni et al., 2005), the mechanism(s) eliciting these aberrations are not completely understood. Moreover, the integrity of cytoskeletal structure is crucial for PRL-mediated pre- and postpartum mammary gland development (Zoubiane et al., 2004), but the impact of HG on the cytoskeletal proteins is not clearly understood. Therefore, we hypothesize that reduced lipogenesis in the HG-exposed mammary gland is induced by anomalies in the cytoskeletal architecture. In this study, we intended to quantify the effect of HG on the cytoskeletal protein abundance during the pregnancy-to-lactation transition in the rat mammary gland.

METHODS

Animals and Treatment Conditions

The use of female Sprague-Dawley rats (Taconic Farms, Germantown, NY) and associated research protocols were reviewed and approved by NASA’s Ames Research Center Institutional Animal Care and Use Committee as reported earlier (Lintault et al., 2007; Patel et al., 2008; Plaut et al., 2003). On gestation day 9 (G9) animals were randomly assigned to either the control group or one of three experimental conditions. These experimental conditions were pregnant (G20, n=4-6), postpartum (lactation) day 1 (P1, n=4-6), and postpartum day 3 (P3, n=4-6). During the period of observation, the dams were individually housed in maternity cages and maintained under standard colony conditions (12:12 light/dark cycle [0600:1800]; 21±1°C at 30-50% humidity). The HG dams were placed on NASA Ames Research Center’s 24 ft centrifuge on day G9 and allowed to acclimatize to continuous 2xg force for 48 h (Lintault et al., 2007; Patel et al., 2008; Plaut et al., 2003). During the acclimation phase, animal behavior, feeding, body temperature, and weights were observed and monitored. The stationary control (SC) dams that were exposed to 1xg were housed in the same room as the HG rats and placed in identical cages to ensure that all groups were exposed to the same environmental conditions (light, sound, temperature, and humidity). The HG (G20, P1, and P3) experimental animals and their respective stationary controls were humanely sacrificed using isoflurane gas. Abdominal mammary gland tissue was then collected as described previously (Patel et al., 2008). The animals from SC and HG groups were sacrificed at the same time in the circadian cycle. During the experimental period, the centrifuge was paused on a daily basis for about an hour to clean cages and replenish food and water, as well as to monitor animal behavior and overall health.

Immunohistochemistry

The tissue for immunohistochemistry was initially fixed in 10% neutral-buffered formalin, and later embedded in paraffin. Thereafter, the preparation of tissue sections (5 µm thickness) and the immunohistochemistry staining protocol were similar to that described elsewhere (Bruxvoort et al., 2007; Cao et al., 2013). Staining for each antibody was optimized using Ventana’s automated staining system (Discovery XT, Ventana Medical Systems, Tucson, AZ) and each slide was simultaneously stained with two primary antibodies. The primary antibodies utilized were rabbit monoclonal anti-alpha-smooth muscle actin (RB 9010-P0, Neomarkers, Fremont, CA) and mouse monoclonal anti-alpha-tubulin (DM1A, Sigma, St. Louis, MO) on one slide. The other slide was stained with rabbit anti-cytokeratin (180052, Life Technologies, Grand Island, NY) and mouse monoclonal anti-vimentin (18-0052, Invitrogen, Camarillo, CA) antibodies. Reactions were observed on the above double-antibody stained slides by incubating with anti-mouse/anti-rabbit multimers [Horseradish peroxidase (Diaminobenzidine), Alkaline phosphatase (Liquid permanent red)] (Ventana Medical Systems, Tucson, AZ). Hematoxylin was utilized as a nuclear-specific counterstain.
Imaging and Multi-Spectral Analysis

Digital micrographs were obtained (10X and 40X magnifications) from each of the G20, P1, and P3 slides using a Brightfield microscope (Nikon Eclipse 80i, Nikon Corporation, Japan). Thereafter, quantitative immunohistochemistry (IHC) analysis was done using the CRi Nuance Multispectral Imaging System (Caliper Life Sciences, MA) as detailed by others (Fiore et al., 2012; Huang et al., 2013). Initially, a spectral library was created for each chromogen as described elsewhere (Fiore et al., 2012; Huang et al., 2013), and then randomly selected lobules (n=3-7 lobules/micrograph; 4 micrographs/slide) were traced using the free-pen tool and analyzed using the automated macros of the Nuance software. Next, the integrated algorithm of the software was used to quantify the staining density per antigen [pixel (px) count] within the traced lobule. The abundance of each immunostain per lobule was then normalized to the hematoxylin counterstain. The Nuance software allowed us to unmix each chromogen and quantify them separately as shown in Figure 1.

Figure 1. Representative images of lobular outlining and unmixing for protein quantification. (a) An overlay image. (b) Overlay image unmixed into blue hematoxylin. (c) Overlay image unmixed into red cytokeratin. (d) Overlay image unmixed into brown vimentin. The unmixed images of a multi-labeled slide enable quantification of individual, as well as overlapping signals within the same outlined lobular border. The scale bar represents 50 µm (10X magnification) in the photomicrographs.

Statistical Analysis

Data within a group [G20 vs P1, G20 vs P3, and P1 vs P3 for HG and SC, respectively] and between groups [HG vs SC within G20, P1, and P3, respectively] were analyzed using Student’s t-test (Graph Pad Prism Software 6.07, San Diego, CA). Data are expressed as mean ± SEM and p<0.05 was considered as significant.
RESULTS

We utilized IHC followed by image analysis using the spectral-spatial features of the CRi Nuance Multispectral Imaging System to investigate the effect of altered gravity on the abundance of cytoskeletal and intermediate filament proteins in pregnant Sprague-Dawley rats.

First, we determined the unique spectral characteristics of each dye/chromophore utilizing the Nuance software and then applied these same spectral parameters to every imaged section for quantification. As illustrated in Figure 1, the ability of the program to distinctly separate counterstain (hematoxylin) from the target analytes allowed us to calculate and express per lobule the amount of each chromophore per unit of DNA staining. We were also able to determine the pixel (px) counts [x,y coordinates] of the paired antigens to find out which ones co-localize and co-express the chromophores.

Expression Levels of Microfilament and Microtubular Proteins

Figure 2 shows multiplexed IHC chromogenic intensity of actin (red) and tubulin (brown) proteins at 10X (Figure 2a), and 40X (Figure 2b) magnifications at G20, P1, and P3 within our targeted region of interest (lobules) in the mammary glands of HG and SC animals. The micrographs demonstrate that the microfilament and microtubular proteins are expressed during pregnancy and lactation in both groups of animals.

Figure 3 provides a quantitative comparison of mammary gland lobular actin and tubulin proteins measured in stationary-control (SC) and hypergravity-exposed (HG) rat groups. A three-fold (p<0.0001) increase in the expression of cytokeratin was noted from G20 (0.084±0.008 px) to P1 (0.27±0.008 px) in the SC animals, followed by a further increase by about 50% (p<0.05) by P3 (0.38±0.04 px) relative to P1 amounts (Figure 5a). A similar trend of time course increase in the expression of cytokeratin from pregnancy to lactation was detected in the HG group (Figure 5a). However, the HG rats expressed about 75% (p<0.0001) more cytokeratin at G20 (0.084±0.008 vs 0.14±0.008 px), as well as approximately 60% (p<0.005) more at P1 (0.27±0.008 vs 0.44±0.05 px) and P3 (0.61±0.05 vs 0.38±0.04 px), respective to their SC cohorts (Figure 3d).

Expression Levels of Intermediate-Filament Proteins

The subcellular distribution of cytokeratin (red) and vimentin (brown) using multiplexed IHC is depicted in Figure 4. Following image acquisition, the nuclear and cytoplasmic analytes were unixed, and each protein was separately quantitated based on the spectral libraries created using Nuance Imaging System. Figure 5 provides a quantitative comparison of mammary gland lobular cytokeratin and vimentin proteins measured in stationary-control (SC) and hypergravity-exposed (HG) rat groups. A three-fold (p<0.0001) increase in the expression of cytokeratin was noted from G20 (0.084±0.008 px) to P1 (0.27±0.008 px) in the SC animals, followed by a further increase by about 50% (p<0.05) by P3 (0.38±0.04 px) relative to P1 amounts (Figure 5a). A similar trend of time course increase in the expression of cytokeratin from pregnancy to lactation was detected in the HG group (Figure 5a). However, the HG rats expressed about 75% (p<0.0001) more cytokeratin at G20 (0.084±0.008 vs 0.14±0.008 px), as well as approximately 60% (p<0.005) more at P1 (0.27±0.008 vs 0.44±0.05 px) and P3 (0.61±0.05 vs 0.38±0.04 px), respective to their SC cohorts (Figure 3d).

There was a stage-specific surge in vimentin quantity in SC rats, such that it increased by about three-fold (p<0.0001) between G20 and P1.
Figure 2. Representative immunohistochemistry (IHC) sections of rat mammary gland from stationary-control (SC) and hypergravity-exposed (HG) animals at day 20 of gestation (G20), postpartum day 1 (P1), and postpartum day 3 (P3), stained for actin (red) and tubulin (brown). (a) Photomicrographs at 10X magnification with scale bar representing 50 µm. Boxes represent images at 40X. (b) Photomicrographs at 40X magnifications with scale bar representing 25 µm and arrows point to actin stained in red.

(0.99±0.03 vs 2.9±0.06 px), as well as between P1 and P3 (2.9±0.06 vs 8.1±0.32 px) (Figure 5c). In contrast, vimentin levels increased by about 50% (p<0.0001) between G20 and P1 (2.9±0.08 vs 4.5±0.28 px), followed by a 300% (p<0.0001) increase between P1 and P3 (4.5±0.28 vs 13.5±1.3 px).
px) in the HG group (Figure 5c). Moreover, the HG animals expressed about three-fold (p<0.0001) more vimentin protein at G20 (0.99±0.03 vs 2.9±0.08 px) compared to SC rats (Figure 5d). Similarly, the amount of vimentin protein at P1 (4.5±0.28 vs 2.9±0.06 px) and P3 (13.5±1.3 vs 8.1±0.32 px) was higher by about 50% (p<0.0001) in HG animals compared to the same stage in SC group (Figure 5d).

Figure 3. Quantitative comparison of mammary gland lobular actin and tubulin proteins measured in stationary-control (SC) and hypergravity-exposed (HG) rat groups following unmixing of chromophores using the CRi Nuance Multispectral Imaging System at day 20 of gestation (G20), postpartum day 1 (P1), and postpartum day 3 (P3). (a) Comparison of actin levels within groups. (b) Comparison of actin levels between groups. (c) Comparison of tubulin levels within groups. (d) Comparison of tubulin levels between groups. Data are shown as Mean ± SEM; * means within and between each group are significantly different (*p<0.01; **p<0.005; ****p<0.0001). The scale has been expanded in insert Figure 3a and 3b to display the relationship of the quantified actin protein.
Figure 4. Representative immunohistochemistry (IHC) sections of rat mammary gland from stationary-control (SC) and hypergravity-exposed (HG) animals at day 20 of gestation (G20), postpartum day 1 (P1), and postpartum day 3 (P3), stained for cytokeratin (red) and vimentin (brown). (a) Photomicrographs at 10X magnification with scale bar representing 50 µm. Boxes represent images at 40X. (b) Photomicrographs at 40X magnifications with scale bar representing 25 µm and arrows point to cytokeratin stained in red.
DISCUSSION

We investigated the spatiotemporal distribution of the cytoskeleton in the mammary gland during mammogenesis and lactogenesis, during a period of altered gravity using a rat model. To our knowledge, the allotment of cytoskeletal elements in the mammary gland during the pregnancy to lactation transition has not been characterized. In addition, the impact of altered inertial force on the distribution of these cytoskeletal components across the physiological phases of pregnancy and lactation has also not been described. We observed that an adjustment of g-load distorts the abundance of microfilaments during physiological phases of both pregnancy and lactation (Figure 3). Our findings, however, show that the abundance of actin proteins during pregnancy and lactation was increased ($p<0.001$) in the mammary gland of HG rats. We noted that the effect of HG on the expression of actin was more pronounced during the transition between...
mammogenesis and lactogenesis. A similar change in actin levels was reported from cancer cells exposed to short term parabolic flight maneuvers (Corydon et al., 2016) and random positioning machine or clinostat exposure (Svejgaard et al., 2015), as well as endothelial cells exposed to HG (Cazzaniga et al., 2014) or microgravity (Jammalek, 2016). Many other studies have reported the immediate effects of gravitation fields on microfilament organization using an array of cells and tissues (Streuli, 1999; Himmelspach et al., 1999; Papaseit et al., 2000; Uva et al., 2002; Hughes-Fulford, 2003; Corydon et al., 2016). In concert with these reports, our results confirm that exposure to chronic HG not only alters the spatiotemporal pattern of expression of the microfilaments, but also markedly increases the quantity of microfilaments across two critically important physiological phases of pregnancy and lactation. However, additional studies are necessary to verify whether the observed changes are due to aberrant synthesis, inhibition of degradation, or redistribution.

We found that the abundance of tubulin protein during pregnancy and early lactation (day 3) was significantly increased in the mammary gland of HG rats, with the difference being higher during lactation compared to pregnancy. Tubulin content does increase in the mammary gland with the morphogenesis of lobulo-alveolar units during pregnancy and milk secretory activation (Brisken et al., 1999; Briskens and O'Malley, 2010; Jamney, 1998; Loizzi and Shao, 1990). The upsurge in tubulin supports the increased synthetic activity of mammary epithelial cells and the intracellular transport of secretory vesicles for exocytosis (Loizzi and Shao, 1990). Therefore, the increased levels of tubulin levels at G20 and P3 in HG animals do suggest that an alteration in g-load induces a dysregulation of tubulin expression at these key phases of mammary gland differentiation. In human neuroblastoma cells, changes in gravitational acceleration modified the modular and spatial arrangements of microtubules, including their orientation (Rosner et al., 2006), polymerization (Portet et al., 2003), and nuclear structural morphology (Crawford-Young, 2006). In rats, a 10% increase in tubulin polymerization occurs during pregnancy, with a further graduated increase to peak by about three-fold at lactation (Loizzi and Shao, 1990). This dynamic adjustment in tubulin content coupled with polymerization during lactation are pivotal for optimum galactopoiesis (Jamney, 1998; Loizzi and Shao, 1990). However, in this study, we did not explicitly assess the impact of HG on tubulin polymerization and protofilament formation in the mammary gland across the studied physiological stages.

Cytokeratins are intermediate filaments found in cells of epithelial origin and are involved in various functions ranging from cellular tensegrity to protein localization to mechano-transduction (Grimm et al., 2006). In addition, these intermediate filaments are the most resilient of the intracytoplasmic filaments and they form a basket-like structure to anchor the nucleus to the cytoplasm. It is known that modification of gravitational acceleration distorts this meshwork of cytokeratin (Vassy et al., 2001), as well as leads to an aberrant expression of cytokeratin genes in endothelial cells (Grosse et al., 2012). Likewise, other studies reported disorganization and rearrangement of intermediate filaments in cancer cells (Ulbrich et al., 2011), endothelial cells (Wehland et al., 2013), and chondrocytes (Aleshcheva et al., 2015). Lactational incompetence, milk content alteration, and atypical lobulo-alveolar patterning is reported with reduction in cytokeratin (Grimm et al., 2006; Hadsell et al., 2007; Sun et al., 2010). However, the specific impact of increased levels of cytokeratin on mammary gland morphology and associated functional output requires elucidation. Overall, our results using the rat mammary gland model are consistent with these earlier studies demonstrating that adjustments in gravity do influence intracellular intermediate filament accretion.

Vimentin is also an intermediate filament that is found in cells of mesenchymal origin. It is part of the intracellular scaffolding that plays an important role in organelle organization, cell attachment, and signaling (Eckes et al., 1998; Satelli and Li, 2011). Like keratins, vimentin is affected by gravitational fields (Eckes et al., 1998; Ulbrich et al., 2011; Ma et al., 2013; Aleshcheva et al., 2015; Maier et al., 2015). On the other hand, overexpression of vimentin has been reported in a wide array of cancers and has been linked to facilitating metastases of tumors (Satelli
and Li, 2011). Our findings are in agreement with earlier studies (Ma et al., 2013; Maier et al., 2015; Ulbrich et al., 2011) and show that the intermediate filament vimentin is significantly elevated in HG-exposed rats during all key stages of mammary gland development. However, additional studies are needed to determine the precise influence of this increased accrretion of vimentin on optimum mammary gland physiology.

Our earlier studies (Casey et al., 2015; Patel et al., 2008) have shown that exposure to altered gravity did not affect gestational length, litter size, or duration of labor or parturition. However, pup survival dropped from 95% at day of parturition to approximately 50% by P3 in HG group (Casey et al., 2012). While milk bands were visible in the HG pups, it was noted that HG dams spent significantly more time nursing compared to their counterparts (Casey et al., 2012; Patel et al., 2008). Interestingly, actin-deficient mice also display precocious lactation and prolonged nursing (Haaksma et al., 2011; Weymouth et al., 2012). However, actin-null mice failed to nurture their offspring effectively (Haaksma et al., 2011; Weymouth et al., 2012). Similar to the HG pups, pups from actin-null mice fail to gain weight and over 60% postnatal mortality was reported (Haaksma et al., 2011; Weymouth et al., 2012). Cross-fostering of both HG and actin-null mice from the day of parturition to their respective non-manipulated controls increased pup survival in both groups to about 90% (Casey et al., 2012; Haaksma et al., 2011; Weymouth et al., 2012). This suggests that the stunted growth noted in HG and actin-deficient pups was not due to an anomaly in the pups but associated with impaired lactation triggered by the disruption of cytoskeleton in the dam’s mammary gland.

Previous studies established that body and mammary gland weights, along with fat stores, were significantly reduced in rats exposed to altered gravity (Casey et al., 2012; Patel et al., 2008). Notably, a similar phenotype has been previously described in PRLR-knockout mice (Freemark et al., 2001). Our earlier study revealed aberrant levels of PRLR, together with atypical spatiotemporal expression pattern in a number of metabolically important organs of animals exposed to altered gravity (Casey et al., 2012; Patel et al., 2008). Moreover, it is established that an integral cytoskeletal microenvironment is necessary to orchestrate the PRL-driven mammary cell differentiation and metabolic homeostasis via the cognate receptor, PRLR (Oakes et al., 2006; Zoubiane et al., 2004). Therefore, the atypical spatiotemporal profile of PRLR observed in our earlier studies (Casey et al., 2012; Patel et al., 2008), is plausibly triggered by the aberrant cytoskeleton architecture observed in the present study. Similarly, the inability to reverse the lactational defect observed in HG-exposed dams through supplementation with lactogenic hormones (Patel et al., 2008) might be partially explained by the failure of the impaired cytoskeleton found in this investigation to constitutively transduce the downstream lactogenic signal.

CONCLUSION

Previous studies attributed the reduced postpartum mammary metabolic output and increased pup mortality rates to deviant PRL secretion and abnormal PRLR expression. The current study reveals that the atypical cytoskeletal architecture may be triggering the reduced postpartum mammary metabolic output in rats exposed to altered gravity. We hypothesize that the impaired cytoskeleton significantly modifies the microenvironment and leads to asynchronous lactogenic signaling by altering the expression of PRLR in the mammary gland.

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