Gravitational and Space Research

Instructions to Authors

Brief Overview:

The journal of the American Society for Gravitational and Space Research (ASGSR), *Gravitational and Space Research*, publishes quality, peer reviewed manuscripts in several categories. Manuscripts should be self-contained, and all conclusions substantiated and supported by results in the form of figures and/or tables. Authors are held to standards of writing (American English) for clarity and material appropriate for the *Gravitational and Space Research (GSR)* journal. Subject matter can include any topic within the following broad categories: the impact of gravity and changes in the gravity vector on biology, spaceflight research (ISS and Shuttle), satellite payloads, advanced life support, planetary and orbital analog research, suborbital research, parabolic flight, sounding rockets, high altitude balloons, astrobiology, plus hardware development, mechanobiology, and other disciplines exploring the interface of biology and engineering technology. Brief summaries of manuscript types and guidelines for each category are below; detailed instructions and templates follow.

I. Short Communications.

Short communications are submissions typically 2 - 3 formatted pages in length (1000 – 2000 words, excluding references). These submissions are to be comprised predominantly of preliminary data for a larger study or a brief report to support work of a larger nature. It may be beyond the scope of these submissions for further experimentation, but a reviewer may request additional explanation of the presented data and hold the authors to appropriate conclusions for those data.

II. Methods papers.

Methods papers are manuscripts typically 3 - 6 pages in length. These manuscripts are comprised of data and protocols that support flight experiments or ground control experiments, of protocols in support of fundamental studies exploring biological responses to altered gravitational environments, and to biological responses to space and planetary analogs. The manuscripts should contain sufficient detail to enable a reader to replicate the protocol. Reviewers should particularly address shortfalls of detail, validation of protocols, and inconsistencies in any aspect of presentation. Figures may include illustrations of procedures and set-up and should include data that verify the efficacy of the procedures.

III. Research papers.

Research papers are manuscripts of typically 8 - 15 pages in length and, although there is no strict limitation to size, a reviewer may address extremes of brevity or length as appropriate to conveying the information. These manuscripts present original research of interest to the gravitational and space research community

IV. Review articles.

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, to be peer-reviewed as any other paper. A review article will be judged principally for accuracy of information and citation and appropriate scope and relevance of the subject of the article.

Detailed Instructions:

Format

The same basic format is used for each type of article. Consult an archived issue of *Gravitational and Space Biology*, as well as the instructions below, for guidance on formatting, organizing, and preparing references, figures, tables, and legends. An article must have a brief abstract that summarizes the principal conclusions of the paper. Manuscripts are submitted electronically as single column, double spaced Word documents, and figures as separate, individual documents. Details are provided below.
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.

Cover page – In a separate page, include the title, suggested running head (not to exceed 60 characters, including spaces), the full names and affiliations of all authors, and detailed contact information for the Corresponding Author: name, address, e-mail, telephone number.

The remaining sections proceed without page breaks

Title – Use a descriptive title (not to exceed 200 characters, including spaces).

Authors – Provide the complete names and affiliations of all authors; indicate the corresponding author.

Abstract – Summarize the principal approach and conclusions of the paper. Abstracts are not to exceed 150 words in Short Communications and 250 words in all others.

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. The Short Communication papers are not required to contain subdivisions, other than a short abstract, but may be organized into subsections at the discretion of the authors.

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).

- 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: author(s): last name(s) comma followed by initial(s) and a period comma before next author; year of publication followed by a period; article title in sentence case, followed by a period; journal title (unabbreviated and italicized), followed by volume number, issue number in parenthesis (if applicable), a colon, and page numbers. Previous issues can be used as a guide, and an EndNote™ style template can be downloaded at the website. Two examples are provided below:

  Journal Article:

  Book:

Figures – Figures are submitted as separate graphic files. Resolution must be 300dpi

- Number Figures consecutively as they are used in the text.
- 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. For submission, provide Figure Legends at the end of the body of the manuscript, following the Reference section.
- Designate figure sections with letters and explain all symbols and abbreviations that are used in the figure.

Tables – Can be submitted as embedded in the text of formatted manuscript in an appropriate location, or subtended to the end of the manuscript.
Instructions to Authors

- 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. For submission, provide Tables and Table Legends at the end of the body of the manuscript, following the Figure Legends.
- Tables should be constructed in Word or Excel with the general format:

<table>
<thead>
<tr>
<th>Pressure (kPa)</th>
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<th>Comments</th>
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<td>101 – 70</td>
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<td>tropical / temperate / taiga biome - many examples of human habitation</td>
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<td>70 – 50</td>
<td>3000 – 5500</td>
<td>tundra / alpine biome - few examples of human habitation</td>
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<td>50 – 30</td>
<td>5500 – 9000</td>
<td>extreme terrestrial elevations - humans require supplemental oxygen</td>
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<td>30 – 5</td>
<td>9000 – 27000</td>
<td>plants can survive as long as temperature is mediated and water is available</td>
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Abbreviations or Other Standards

- 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.
- Use spaceflight (one word) rather than space flight (two words).
- Any acronyms that are used in the manuscript must be defined at first mention.

Manuscript Review and Preparation of Final Version

Prior to publication, manuscripts are reviewed by the editor assigned to an author’s article and, generally, by two scientific reviewers.

If reviewers recommend only minor textual changes, the editor may choose to make these changes and accept the manuscript essentially as submitted. The editor then sends the accepted manuscript to the journal’s publishing editor. Page proofs are provided to the authors for review prior to the journal going to press.

Deadlines

Editors will inform authors of their deadlines. Deadlines will have limited flexibility, but under no circumstances will the publication of the journal be delayed to accommodate late manuscripts.
Authorship Statement

Authorship of articles implies that an individual has made a substantial contribution to the article both in terms of the design of the study or collection/evaluation of data and with regard to the intellectual content of the manuscript.

Conflict-of-Interest Statement

Reviewers recruited for the evaluation of manuscripts being considered for publication in *Gravitational and Space Research* will be held to Conflict of Interest standards comparable to those required of NSF and NASA panelists. You may be considered in conflict if:

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- Employment as faculty at the submitting institution or as a consultant or advisor to the institution.
- Previous employment with the institution within the last 12 months.
- Being considered for employment at the institution.
- Hold any office, governing board membership, or relevant committee chair in the institution.
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- Past or present association as thesis advisor or thesis student.
- Past or present association as post-doctoral advisor or postdoctoral student within the past 5 years.
- Collaboration on a project or on a book, article, report, or paper within the last 2 years.
- Co-editing of a journal, compendium, or conference proceedings within the last 1 year.

Published Statement of Human and Animal Rights:

Research involving Human and Animal Subjects must have been approved by the author’s institutional review board. Authors must include in the Methods section a brief statement identifying the institutional and/or licensing committee approving the experiments. For experiments involving human subjects, authors must also include a statement confirming that informed consent was obtained from all subjects. All experiments involving human subjects must have been conducted according to the principles expressed in the Declaration of Helsinki. If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must explain the rationale for their approach, and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study. When reporting experiments on animals, authors should be asked to indicate whether the
institutional and national guide for the care and use of laboratory animals was followed. For research using Recombinant DNA, physical and biological containment must conform to National Institutes of Health guidelines or those of a corresponding agency.

**Published Statement of Informed Consent:**

The general requirements for informed consent conform to guidelines and requirements outlined by the National Science Foundation [http://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf](http://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf) and Health and Human services [http://answers.hhs.gov/ohrp/categories/1566](http://answers.hhs.gov/ohrp/categories/1566). No investigator may involve a human being as a subject in research covered by this policy unless the investigator has obtained the legally effective informed consent of the subject or the subject's legally authorized representative. An investigator shall seek such consent only under circumstances that provide the prospective subject or the representative sufficient opportunity to consider whether or not to participate and that minimize the possibility of coercion or undue influence. The information that is given to the subject or the representative shall be in language understandable to the subject or the representative. No informed consent, whether oral or written, may include any exculpatory language through which the subject or the representative is made to waive or appear to waive any of the subject's legal rights, or releases or appears to release the investigator, the sponsor, the institution or its agents from liability for negligence.

**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.
# Table of Contents

## General Information

**Editorial Board** ......................................................................................................................... iii

**Instructions to Authors** ................................................................................................................. iv

**Journal Policies** ................................................................................................................................. vii

## Table of Contents ................................................................................................................................ 1

### Research Articles

**Housing in the Animal Enclosure Module Spaceflight Hardware Increases Trabecular Bone Mass in Ground-Control Mice**  
Shane A. Lloyd, Virginia S. Ferguson, Steven J. Simske, Alexander W. Dunlap, Eric W. Livingston, and Ted A. Bateman ................................................................. 2

**The Effects of Spaceflight on Mucin Production in the Mouse Uterus**  
Allan D. Forsman and Heath A. Nier ................................................................. 20

**Spaceflight Effects and Molecular Responses in the Mouse Eye: Preliminary Observations After Shuttle Mission STS-133**  
Susana B. Zanello, Corey A. Theriot, Claudia Maria Prospero Ponce, and Patricia Chevez-Barrios ................................................................. 29

**Effects of Underwater Arm-Cranking Exercise on Cardiac Autonomic Nervous Activity**  
Kumiko Ono, H. Kuniyoshi, and Y. Tanigaki ................................................................. 47

**Development in Altered Gravity Influences Height in Dictyostelium**  
Morris A. Benjaminson, James A. Gilchrist, and Stanley Lehrer ................................................................. 51

**A Computational Study of the Mechanics of Gravity-induced Torque on Cells**  
Ioannis Haranas, Ioannis Gkigkitzis, and George D. Zouganelis ................................................................. 59

**The Effects of Gamma and Proton Radiation Exposure on Hematopoietic Cell Counts in the Ferret Model**  
Jenine K. Sanzari, X. Steven Wan, Gabriel S. Krigsfeld, Andrew J. Wroe, Daila S. Gridley, and Ann R. Kennedy ................................................................. 79

**Preliminary Species and Media Selection for the Veggie Space Hardware**  
Gioia Massa, Gerard Newsham, Mary E. Hummerick, Janicce L. Caro, Gary W. Stutte, Robert C. Morrow and Raymond M. Wheeler ................................................................. 95

### Review Articles

**Mammalian Reproduction and Development on the International Space Station (ISS): Proceedings of the Rodent Mark III Habitat Workshop**  
April E. Ronca, Joshua S. Alwood, Ruth K. Globus, and Kenneth A. Souza ................................................................. 107

## Index of Authors ...................................................................................................................................... 124
Housing in the Animal Enclosure Module Spaceflight Hardware Increases Trabecular Bone Mass in Ground-Control Mice

Shane A. Lloyd\textsuperscript{1}, Virginia S. Ferguson\textsuperscript{2,3}, Steven J. Simske\textsuperscript{2,4}, Alexander W. Dunlap\textsuperscript{5}, Eric W. Livingston\textsuperscript{6}, and Ted A. Bateman\textsuperscript{6}

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ABSTRACT

During spaceflight, mice are housed in specially designed cages called the Animal Enclosure Module (AEM). Utilization of this flight hardware may affect the skeletal properties of housed animals, independent of microgravity considerations. To address this issue, we studied the effect of 13 days of AEM housing versus standard vivarium enclosure on female C57BL/6J mice (n=12/group). The effects of AEM housing were most pronounced in the trabecular compartment. AEM mice had 44% and 144% greater trabecular bone volume fraction and connectivity density, respectively, versus vivarium. A similar response was seen at the proximal humerus. We noted a decrease in proximal tibia osteoclast surface (-65%) and eroded surface (-73%) for AEM versus vivarium, while tibia trabecular mineralizing surface (MS/BS) was nearly three-fold greater. Surprisingly, there was also decreased osteoblast surface, as well as lower osteoid volume, surface, and thickness at this site. The effects of AEM housing on femur cortical bone were modest: there was greater periosteal MS/BS, with no effect at the endocortical surface, and lower femur stiffness. Taken together, we have demonstrated significant effects of AEM housing on ground control mice, particularly in the trabecular bone compartment. These findings suggest that an early increase in bone formation, perhaps due to altered behavior and loading in this unique housing environment, was followed by decreased bone formation and resorption as the animals adapted to their new environment. Characterization of spaceflight animal housing is critical to elucidating the true effects of microgravity on skeletal parameters and for the proper selection of ground-based controls.

INTRODUCTION

The “weightless” environment encountered during spaceflight complicates the traditional concerns involved in animal housing, including nutrient delivery and waste management. Spaceflight experiments utilizing mice or rats

Key words: Animal Enclosure Module; Housing; Mice; Bone; Spaceflight; Microcomputed Tomography; Histomorphometry; Mechanical Testing; Ground Control

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require habitats that are specifically designed for this environment. During studies on the space shuttle, rodents are housed in a specially designed stainless steel meshed cage called the Animal Enclosure Module (AEM) (Brooks, 1981). Despite the retirement of the shuttle program in 2011, plans are in place to adapt the AEM for use on the International Space Station (ISS). AEMs are equipped with systems that provide food and water *ad libitum* (Zerath et al., 2002). In addition, these flight enclosures have a constant airflow mechanism designed to move free-floating animal waste towards an exhaust filter. AEMs allow for efficient containment of animals in the confines of the crew cabin, while also satisfying their homeostatic demands. The first test flight of the AEM was during shuttle mission STS-8 in 1983 (Smith et al., 1987). Since then, AEMs have been successfully utilized on over 20 missions involving both rats and mice.

Despite their demonstrated utility, AEM housing may have an effect on the physiology of the rodents contained within. Beyond consideration of the spaceflight environment, a non-standard cage environment like that found with AEMs may lead to unexpected changes in animal behavior that could complicate experimental results. These considerations are especially important in studies examining the skeletal effects of microgravity. In this case, even subtle changes in animal loading can distort experimental findings. For example, a study by Morey-Holton and colleagues revealed that group housing of rats in AEMs reduced their skeletal response to spaceflight by as much as 80% (Morey-Holton et al., 2000).

In the present study, we compared the effects of 13 days of AEM housing on various bone compositional, microarchitectural, and histological parameters. We documented significant effects of the AEM on the skeletal properties of mice, particularly in the trabecular bone compartment. This study was conducted as part of a ground-based parallel of an animal payload on space shuttle flight STS-108. Characterization of animal housing is important so that results from spaceflight experiments can be more reliably compared to ground-based controls and studies from other missions.

**MATERIALS AND METHODS**

*Ethics Statement*

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol for this study was approved by the Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) Kennedy Space Center (Protocol #01-028-1).

*Animals*

This thirteen-day experiment, conducted on the ground, modeled the flight experiment profile of the CBTM-01 payload on STS-108. 64 day-old female C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME, USA) were utilized for this study. Mice were assigned to standard vivarium cages or AEM flight hardware at n=12/group. A group of baseline mice (n=12) was sacrificed on day 0. AEM and vivarium mice received an intraperitoneal injection of the fluorescent bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study.

*Flight Hardware & Housing*

On the space shuttle, mice are maintained in AEM hardware provided by NASA Ames Research Center (Moffett Field, CA, USA) (Figure 1). The AEMs provide rodent food (NASA Rodent Diet TD97071, Harlan-Teklad; Wisconsin, USA) and water *ad libitum* (Zerath et al., 2002). Because urine and feces are free to float around the cage in microgravity, the AEMs have a constant airflow mechanism designed to move waste towards an exhaust filter. The dimensions of the AEM are 24.50 x 43.69 x 51.05 cm and it weighs approximately 27.2 kg with food, water, and animals. The total floor space with the water box installed is 645 cm$^2$. The AEM can support up to five adult rats or ten adult mice. For the present study, housing density was within NIH guidelines with approximately 81 cm$^2$ floor area per mouse (eight mice per AEM, with a divider separating half the mice). AEMs were contained within the Orbiter Environmental Simulator (OES) at NASA’s Life Science Support Facility (“Hangar L”) at Cape Canaveral Air Force Station (Cape Canaveral, FL, USA). The OES creates an
environment that mimics the temperature, humidity, and CO$_2$ levels within the cabin of the space shuttle. The parameter of primary concern was atmospheric CO$_2$, which averaged more than 3000 ppm during the STS-108 flight. This level is normal in spacecraft and is approximately ten times that of a well-ventilated room on Earth. The constant airflow mechanism described above was activated for this ground-based study, although it was not able to clear waste in normal gravity. Four internal lamps provided an average 14 lux of illumination with timers to provide 12-hour light/dark cycles.

The vivarium-housed animals were maintained in the Animal Care Facility at Hangar L with standard environmental conditions, feeding mechanisms, and a 12-hour light/dark cycle. Exact space shuttle environmental conditions (i.e., temperature, humidity, CO$_2$) were not reproduced for vivarium-housed mice. Each cage had a floor area of 522 cm$^2$ and animals were group housed (4 mice/cage; 130 cm$^2$ of floor area per mouse) with access to the same rodent diet and water ad libitum as their AEM-housed counterparts.

Necropsy

Baseline animals were sacrificed at the start of the study. After 13 days of housing, vivarium and AEM-housed mice were sacrificed. All animals were weighed and then anaesthetized with isoflurane (2%). Mice were then sacrificed by exsanguination via cardiac puncture and cervical dislocation. Hindlimbs and forelimbs were removed and the tibia, femur, humerus, and lumbar vertebrae of each animal were isolated and cleaned of all non-osseous tissue. The left femur,
required for mechanical testing and mineral composition analysis, was allowed to air-dry. The right femur, left and right tibia, right humerus, and L5 lumbar vertebrae were fixed in a 10% neutral buffered formalin solution for 48 hours, rinsed with distilled water, and stored in 70% ethanol. These bones were utilized for microcomputed tomography analysis, histology, and histomorphometry as described below.

**Microcomputed Tomography**

Trabecular bone architecture was analyzed using microcomputed tomography (µCT20; Scanco Medical AG; Brüttisellen Switzerland) with an isotropic voxel size of 9 µm with scan settings of 55 KVP, 145 mA, and 200 ms integration time. Trabecular microarchitecture was analyzed with Scanco software immediately distal to the epiphyseal plate in the right proximal tibia and humerus. Trabecular bone evaluation was performed on 100 slices (0.9 mm total) for each tibia and 70 slices (0.6 mm total) for each humerus, producing three-dimensional images for analysis. Bone morphometric parameters were then quantified using Scanco software. In accordance with published guidelines (Bouxsein et al., 2010), trabecular bone parameters included trabecular bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and structure model index (SMI).

**Cortical Histomorphometry**

After ethanol fixation, the right femurs were air-dried and embedded in non-infiltrating Epo-Kwick epoxy (Buehler; Lake Bluff, IL, USA). These epoxy disks were sectioned in half with a low-speed saw (Buehler; 12.7 cm x 0.5 mm diamond blade) at the mid-diaphysis of the femur. The proximal half was wheel-polished to a flat, smooth surface with 600-, 800-, and 1200-grit carbide paper, followed by a cloth impregnated with 6 µm diamond paste. This allowed micrographs at 50× magnification to be taken of the femur cross-section under a blue light (400 nm). Green Calcein labels were visualized, indicating the bone formation sites present during the study. Quantitative histomorphometric analysis was performed using these photographs and SigmaScan Pro software (SPSS; Chicago, IL, USA).

Measurements of bone morphology (Dempster et al., 2013) included tissue volume (TV) enclosed by the periosteal bone surface (Ps.BS) and volume of the marrow cavity (Ma.V) enclosed by the endocortical bone surface (Ec.BS). Bone volume (BV) was calculated as TV - Ma.V. Cortical thickness (Av.Ct.Th) was measured at the medial, lateral, posterior, and anterior location of the femur diaphysis and averaged.

Due to the absence of double Calcein labels, we were not able to accurately determine mineral apposition rate (MAR) or bone formation rate (BFR) (Foldes et al., 1990). As a result, we used the mineralizing surface (MS/BS) as an index of bone formation as described previously (Grimston et al., 2011). The linear content of the Calcein labeled perimeter was defined as mineralizing surface (MS) and normalized to bone surface (BS) at both Ps and Ec surfaces (i.e., Ps.MS/BS and Ec.MS/BS).

The proportional endocortical eroded surface (Ec.ES/BS) was measured by quantifying the portion of the non-labeled surface with a rough/ruffled border and dividing it by BS.

**Biomechanical Properties**

In order to simulate in vivo properties, the air-dried left femurs were rehydrated in phosphate-buffered saline for 90 minutes prior to evaluation (Broz et al., 1993). Three-point bending tests were performed using an Instron 5582 (Instron Corporation; Norwood, MA, USA). Femurs were tested to failure with a 9 mm span length and a deflection rate of 5 mm/min. All bones were tested in the same orientation: the single-point load was applied mid-diaphysis on the anterior surface. The maximal force (Fm; N) and deflection at Fm (δm; mm) were measured for all mechanically tested bones. These two properties were also determined at the elastic limit (Fe, δe) and the failure point. Stiffness (N/mm) was calculated from elastic force/elastic deflection (Fe/δe).

Two-dimensional, cross-sectional moments of inertia of the right femur mid-diaphysis (Iₓ and Iᵧ; mm⁴) were also calculated. These values were determined using micrographs and the assumption that the periosteal and endocortical surfaces were
Lloyd et al. -- Skeletal Effects of the Animal Enclosure Module

in the approximate shape of concentric ellipses (Simske et al., 1992).

Right femurs prepared for histomorphometric analysis were utilized for testing the material properties of the femur diaphysis by microhardness indentation. Three microhardness indents were placed in extant bone within each sectioned and polished femur cross-section using a pyramid-shaped Vicker’s diamond indenter (Fischer Scope-H1100 and WINHCU 1.3 software, Fischer Technology; Windsor, CT, USA) with a 50 g load for 10 seconds. In order to minimize edge effects, one indent length was maintained between the indent site, sample edges, and visible lacunae. Pyramid diagonal lengths were measured (250 μm), and the Vicker’s hardness number (VHN; kgf/mm²) was calculated using the formula: VHN=(2Fsin(x/2))/d², where F=applied load, x=pyramid angle (136°), and d=average measure of the two diagonal lengths.

Osteoblast and Osteoclast Identification

Following microCT analysis, the right tibias and L5 vertebrae were decalcified using a formic acid solution (Immunocal; Decal Chemical Corporation, Talman, NY, USA) and embedded in a methylmethacrylate resin (ImmunoBed; Polysciences, Warrington, PA, USA) as described previously (Chappard et al., 1987; Erben, 1997). The samples were cut into sagittal sections with a thickness of 3 μm using a microtome (Leica Microsystems, model RM2165; Witzlar, Germany). Each slide was stained with TRAP using a commercial kit (Sigma; St. Louis, MO, USA) to identify osteoclasts and counterstained with hematoxylin to identify osteoblasts. These sections were also used to quantify osteoid volume normalized to total volume (OV/TV), osteoid surface normalized to bone surface (OS/BS), and osteoid thickness (O.Th).

Trabecular Histomorphometry

The left tibias were embedded in a methylmethacrylate resin (Osteo-Bed; Polysciences, Warrington, PA, USA) and cut into sagittal sections with a thickness of 5 μm using a tungsten carbide blade. Slides were left unstained. Quantitative histomorphometric analysis was performed using SigmaScan Pro software on the micrographs captured at 10× magnification under a UV light (400 nm). Histomorphometric evaluation was performed throughout the metaphysis, starting approximately 0.25 mm distal from the growth plate and extending a further 0.5 mm. MS/BS and ES/BS were quantified in the same manner as for cortical bone.

Bone Mineral Composition

Mineral-content analysis was performed on left femurs fractured during mechanical testing. Prior to analysis, the enlarged ends of the femurs were separated where the distal and proximal metaphysis joins the diaphysis. Mineral content data was obtained separately from these bone ends and the diaphysis itself. A properly calibrated analytical scale (Mettler Toledo UMT2; Columbus, OH, USA) was used for all measurements. Dry mass (Dry-M) was measured after heating the bones to 105°C for 24 hours. Mineral mass (Min-M) was measured after the bones had been heated at 800°C for an additional 24 hours. Organic mass (Org-M) was calculated as the difference between the two (Org-M = Dry-M - Min-M). Percent mineralization was calculated as: %Min = (Min-M)/(Dry-M)*100.

Serum Chemistry

At sacrifice, samples of whole blood were collected by cardiac puncture and serum was separated. The concentration of various bone turnover markers was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits. ELISAs were performed for the bone resorption marker tartrate-resistant acid phosphatase 5b (TRAP5b) (ImmunoDiagnostic Systems Inc; Fountain Hills, AZ, USA), as well as the bone formation markers osteocalcin and alkaline phosphatase (Biomedical Technologies Inc; Stoughton, MA, USA). All ELISA procedures were performed according to the manufacturers’ protocols. Calcium and phosphorus levels were also determined using a Hitachi 717 Automatic Chemistry Analyzer (Roche Diagnostics; Indianapolis, IN, USA).

Statistics

Statistics were completed using Prism 5.0 software (GraphPad Software Incorporated; La Jolla, CA, USA). Statistical comparisons were made via one-way-ANOVA between baseline,
vivarium, and AEM groups with a Student Newman-Keuls post-hoc test. Type I error was set at 95% (p<0.05). All data are reported at mean ± standard error of the mean (SEM). Unless otherwise indicated, all reported changes represent significant differences (p<0.05). Generally, differences between vivarium and AEM groups will be reported and discussed. Differences in relation to baseline control mice are presented to aid in determining if differences between vivarium and AEM are related to normal growth or hardware effects.

RESULTS

Animal Mass

There was no significant difference between any of the groups with respect to total body mass at the start of the study (baseline: 17.5 ± 0.1 g, vivarium: 17.5 ± 0.2 g, AEM: 17.7 ± 0.1 g) or at the end of the study (vivarium: 18.6 ± 0.2 g, AEM: 18.6 ± 0.2 g) (p>0.05 for all).

Mechanical, Material, and Structural Assays

Mechanical stiffness was 18% lower for AEM-housed mice versus vivarium control (p<0.05) (Table 1). There were no other significant differences between AEM and vivarium for any of the parameters obtained from the mechanical, material, and geometric assays that were conducted. Stiffness, elastic force, and maximal force were similarly and significantly greater in vivarium and AEM-housed animals when compared to baseline (p<0.05). Analysis of calculated resistance to torsion revealed significantly greater I_max in AEM-housed mice compared to baseline (p<0.05), with no difference for vivarium. There was no difference in microhardness values between baseline mice and either housing environment.

Table 1. Mechanical, material, and structural properties of the femur. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with ¥ or ¥, respectively (p<0.05).

<table>
<thead>
<tr>
<th>Property</th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiffness (N/mm)</td>
<td>42.3 ± 2.5¥¹</td>
<td>59.1 ± 2.7¥</td>
<td>50.3 ± 2.1¥</td>
</tr>
<tr>
<td>Elastic Force (N)</td>
<td>8.6 ± 0.4¥</td>
<td>11.2 ± 0.4</td>
<td>11.4 ± 0.3</td>
</tr>
<tr>
<td>Maximal Force (N)</td>
<td>10.3 ± 0.3¥</td>
<td>12.6 ± 0.3</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td>Failure Force (N)</td>
<td>8.6 ± 0.4</td>
<td>7.9 ± 0.6</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>I_max (mm⁴)</td>
<td>184 ± 9¥</td>
<td>213 ± 8</td>
<td>238 ± 6</td>
</tr>
<tr>
<td>I_min (mm⁴)</td>
<td>97 ± 4</td>
<td>106 ± 4</td>
<td>113 ± 3</td>
</tr>
<tr>
<td>Micro-Hardness (kgf/mm²)</td>
<td>67.6 ± 1.7</td>
<td>71.5 ± 1.3</td>
<td>72.9 ± 1.3</td>
</tr>
</tbody>
</table>

Cortical Histomorphometry

There were no changes in cortical BV, TV, or Ma.V for AEM versus vivarium (Table 2). As expected due to normal growth, BV and TV were similarly and significantly increased by approximately 5% for both vivarium and AEM versus baseline.

Ps.MS/BS was significantly greater in AEM-housed mice (+21%) when compared to vivarium (Figure 2A), while no effect on Ec.MS/BS was observed (Figure 2B). There was no effect of housing environment on Av.Ct.Th or Ec.ES/BS (Table 2). AEM-housed animals had a 14% higher Av.Ct.Th versus baseline that was not observed in vivarium-housed.
Trabecular Microarchitecture

Animal housing environment had a significant effect on trabecular microarchitectural parameters at the proximal tibia, as shown in a representative microCT image (Figure 3). Compared to vivarium, mice housed in AEMs demonstrated greater BV/TV (+44%; Figure 4A) and Conn.D (+144%; Figure 4B). As shown in Table 3, there were also significant differences in SMI (-12%), Tb.N (+11%), and Tb.Sp (-10%) at the proximal tibia for AEM versus vivarium. There was no difference in Tb.Th for AEM versus vivarium. Compared to baseline, AEM-housed animals had 61% greater BV/TV and 193% greater Conn.D. There were no significant differences between baseline and vivarium for any parameters measured at the proximal tibia.

Analysis of trabecular microarchitecture at the proximal humerus revealed that AEM-housed mice had an 18% greater BV/TV than vivarium controls, with a non-significant trend suggesting greater Conn.D (Table 3). These differences were due a decrease in trabecular BV/TV and Conn.D in vivarium-housed mice compared to baseline.

Trabecular Histomorphometry

Trabecular MS/BS was 295% greater for AEM-housed mice versus vivarium (Figure 4C). Osteoid volume, osteoid surface, and osteoid thickness were all significantly lower for AEM-housed mice compared to both vivarium-housed and baseline (Table 4). Eroded surface was 73% lower for AEM-housed versus vivarium.

Table 2. Femur cortical histomorphometric parameters. Data was obtained from femur mid-diaphysis cross-sections collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Vivarium and AEM mice received an injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. BV=bone volume, TV=tissue volume, Ma.V=medullary volume, Av.Ct.Th=average cortical thickness, Ec=endocortical, Ec.ES=endocortical eroded surface, BS=bone surface. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV (mm³)</td>
<td>0.65 ± 0.01</td>
<td>0.68 ± 0.01</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>TV (mm³)</td>
<td>1.54 ± 0.02</td>
<td>1.60 ± 0.02</td>
<td>1.59 ± 0.01</td>
</tr>
<tr>
<td>Ma.V (mm³)</td>
<td>0.90 ± 0.01</td>
<td>0.91 ± 0.02</td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>Av.Ct.Th (µm)</td>
<td>170 ± 6</td>
<td>176 ± 5</td>
<td>194 ± 5</td>
</tr>
<tr>
<td>Ec.ES/BS (%)</td>
<td>-</td>
<td>19.9 ± 2.9</td>
<td>24.5 ± 2.3</td>
</tr>
</tbody>
</table>

For AEM-housed mice, tibia trabecular Ob.S/BS was 79% lower than vivarium (Figure 5A), while Oc.S/BS of AEM mice was 65% lower than vivarium (Figure 5B). Compared to baseline, both vivarium and AEM-housed mice had a lower measured osteoblast and osteoclast surface; however, the decrease was greater in magnitude for AEM for both Ob.S/BS (-85% vs. -28%) and Oc.S/BS (-75% vs. -30%).

At the mid-L5 vertebrae, Ob.S/BS was found to be significantly decreased for AEM mice when compared to vivarium (-70%; Figure 5C). Oc.S/BS for AEM mice was 32% lower than their vivarium-housed peers (Figure 5D). There were no differences between baseline and vivarium for either Ob.S/BS or Oc.S/BS. Conversely, AEM-housed animals had a significantly lower Ob.S/BS (-60%) and Oc.S/BS (-30%).
Table 3. Trabecular microarchitectural parameters of the tibia and humerus. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. SMI=structure modeling index, Tb.N=trabecular number, Tb.Th=trabecular thickness, Tb.Sp=trabecular separation, BV/TV=trabecular bone volume fraction, Conn.D=trabecular connectivity density. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal Tibia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMI (no units)</td>
<td>3.08 ± 0.06</td>
<td>2.98 ± 0.06*</td>
<td>2.63 ± 0.07*</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>4.07 ± 0.07</td>
<td>4.01 ± 0.09*</td>
<td>4.44 ± 0.1*</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.036 ± 0.007</td>
<td>0.039 ± 0.006</td>
<td>0.040 ± 0.004</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.247 ± 0.005</td>
<td>0.252 ± 0.006*</td>
<td>0.228 ± 0.006*</td>
</tr>
<tr>
<td><strong>Proximal Humerus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>10.4 ± 0.5V</td>
<td>8.5 ± 0.4*</td>
<td>10.0 ± 0.6*</td>
</tr>
<tr>
<td>Conn.D (1/mm³)</td>
<td>80 ± 7V</td>
<td>53 ± 8</td>
<td>73 ± 9</td>
</tr>
</tbody>
</table>

Table 4. Tibia trabecular histomorphometric parameters. Data was obtained from tibia metaphysis cross-sections collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Vivarium and AEM mice received an injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. OV/BV = osteoid volume normalized to bone volume, OS/BS = osteoid surface normalized to bone surface, O.Th = osteoid thickness, ES/BS = eroded surface. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>OV/BV (%)</td>
<td>4.6 ± 0.4V</td>
<td>4.1 ± 0.3</td>
<td>1.2 ± 0.2*</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>19 ± 1A</td>
<td>18 ± 1</td>
<td>8 ± 1*</td>
</tr>
<tr>
<td>O.Th (µm)</td>
<td>3.1 ± 0.2A</td>
<td>3.0 ± 0.1</td>
<td>2.5 ± 0.1*</td>
</tr>
<tr>
<td>ES/BS (%)</td>
<td>-</td>
<td>4.5 ± 0.6</td>
<td>1.4 ± 0.3*</td>
</tr>
</tbody>
</table>

Mineral Composition

There was no effect of AEM housing on the whole femur dry, organic, or mineral mass (Table 5). Percent mineralization of the femur diaphysis was slightly, but significantly, lower for AEM versus vivarium (-2%), although this was due to a significant increase in diaphysis mineralization for vivarium versus baseline (+2%). Total mineral content of the femur was also greater for vivarium versus baseline (+2%), although the metaphysis mineral content was not. Dry, organic, and mineral mass were similarly and significantly increased for vivarium and AEM versus baseline.
Figure 2. Cortical quantitative histomorphometric parameters. Femur mid-diaphysis cross-sections were collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Vivarium and AEM mice received a single injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. Measured parameters included mineralizing surface normalized to bone surface (MS/BS) at both the (A) periosteal surface (Ps.MS/BS) and (B) endocortical surface (Ec.MS/BS). MS/BS was used as an index of bone formation. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05).

Figure 3. Representative images of trabecular microstructure at the proximal tibia. These images illustrate the greater amount of trabecular bone in mice housed in the Animal Enclosure Module (AEM) versus standard Vivarium housing. Images were selected based on group mean for trabecular bone volume fraction (BV/TV). Images were acquired using a µCT20 (Scanco Medical AG; Brüttisellen, Switzerland) with an isotropic voxel size of 9 μm and represent a three dimensional reconstruction of 100 slices (0.9 mm total) of the tibia, immediately distal to the epiphysial plate.
Figure 4. Trabecular microstructural and histomorphometric parameters. Tibias were collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Microstructural parameters assessed by microCT included (A) trabecular bone volume fraction (BV/TV) and (B) trabecular connectivity density (Conn.D). Vivarium and AEM mice received a single injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. Measured trabecular histomorphometric parameters included (C) trabecular mineralizing surface normalized to bone surface (MS/BS). MS/BS was used as an index of bone formation. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

Table 5. Mineral composition analysis of the femur. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Dry Mass (mg)</td>
<td>28.1 ± 0.5</td>
<td>31.1 ± 0.3</td>
<td>31.3 ± 0.3</td>
</tr>
<tr>
<td>Total Organic Mass (mg)</td>
<td>10.6 ± 0.2</td>
<td>11.3 ± 0.1</td>
<td>11.6 ± 0.1</td>
</tr>
<tr>
<td>Total Mineral Mass (mg)</td>
<td>17.5 ± 0.3</td>
<td>19.8 ± 0.2</td>
<td>19.7 ± 0.2</td>
</tr>
<tr>
<td>Total Mineral Content (%)</td>
<td>62.3 ± 0.3</td>
<td>63.7 ± 0.2</td>
<td>63.0 ± 0.3</td>
</tr>
<tr>
<td>Diaphysis Mineral Content (%)</td>
<td>64.0 ± 0.3</td>
<td>65.5 ± 0.4</td>
<td>64.4 ± 0.2</td>
</tr>
<tr>
<td>Metaphysis Mineral Content (%)</td>
<td>60.1 ± 0.4</td>
<td>61.0 ± 0.5</td>
<td>61.3 ± 0.5</td>
</tr>
</tbody>
</table>
Figure 5. Trabecular osteoblast and osteoclast surface parameters. Histological sections were prepared from the trabecular bone of the tibia and L5 lumbar vertebrae collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. We assessed (A) tibia trabecular osteoblast surface normalized to bone surface (Ob.S/BS) and (B) tibia trabecular osteoclast surface normalized to bone surface (Oc.S/BS). These measurements were also made at the L5 vertebrae (C,D). Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

Serum Chemistry

Serum markers of bone formation were lower for AEM-housed mice when compared to vivarium. Osteocalcin levels in AEM-housed mice were 35% lower than their vivarium-housed counterparts (Figure 6A). The effect of housing environment on alkaline phosphatase levels was more modest: AEM-housed mice had alkaline phosphatase levels 25% lower than vivarium-housed mice (Figure 6B). There was no effect of enclosure type on the bone resorption marker TRAP5b (Figure 6C). Alkaline phosphatase was lower for both vivarium and AEM versus baseline, although the magnitude was greater for AEM (-40%) than vivarium (-20%).

Serum calcium was not affected by cage environment (Figure 6D) and there were no differences in serum calcium for either AEM or vivarium versus baseline. AEM-housed mice did have significantly greater serum phosphorus levels (+20%) when compared to vivarium-housed controls (Figure 5E), although this was due to a decrease in phosphorus for vivarium-housed animals versus baseline. Serum phosphorus for vivarium-housed mice was significantly lower than baseline (-19%), while there was no difference between baseline and AEM.
Lloyd et al. -- Skeletal Effects of the Animal Enclosure Module

Figure 6. Serum markers of bone turnover. Levels of the serum bone formation marker (A) osteocalcin and (B) alkaline phosphatase were assessed. In addition, we measured the serum bone resorption marker (C) tartrate-resistant acid phosphatase 5b (TRAP5b) as well as serum levels of (D) calcium and (E) phosphorus. Blood was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

DISCUSSION

We have demonstrated that ground-based use of the AEM results in significant, albeit complicated, effects on murine skeletal physiology. With only modest effects on cortical bone, the AEM was found to produce its most dramatic effects in the trabecular bone compartment. At the proximal tibia and humerus, we observed significantly greater trabecular bone volume fraction and connectivity density in AEM-housed mice versus vivarium. Histological analysis at the proximal tibia suggests that increased accumulation of trabecular bone mass was likely due to a cessation of bone resorption, as there was a significantly lower osteoclast surface and eroded surface for AEM mice when compared to vivarium. Previous studies have determined that, beginning at approximately 6 weeks of age, C57BL/6J mice begin to continuously lose trabecular bone mass at multiple sites (Ferguson et al., 2003; Halloran et al., 2002). Given the 8-10 week age of these mice, the maintenance of trabecular bone structure at the proximal tibia and decline in trabecular bone structure at the proximal humerus of vivarium mice is not unexpected. Indeed, the greater trabecular bone mass and changes in microarchitectural parameters in AEM versus vivarium mice at multiple sites suggest that a significant hardware effect is present.

Histological analysis also revealed a decrease in osteoblast surface for AEM-housed animals.
versus vivarium at both the proximal tibia and the L5 vertebrae. Osteoid volume, surface, and thickness at the proximal tibia were all significantly lower for AEM-housed mice. There were also lower endpoint levels of serum osteocalcin and alkaline phosphatase for AEM mice. Taken together, these findings point to an overall decline in osteoblast activity and bone formation (Gundberg et al., 2002; Lumachi et al., 2009). This was not the case, however, for periosteal mineralization surface, which was used as an index of bone formation (Grimston et al., 2011), in femur cortical bone (Figure 2A). We also demonstrated a significant increase in mineralizing surface at the proximal tibia (Figure 4C). In considering the seeming incongruence of these histological and histomorphometric findings, it is also important to consider the methodology underlying the quantitative histomorphometric analyses. By administering a single Calcein label at day 0, one is actually visualizing mineralization sites present at any point over the course study, without being able to resolve fluctuations throughout. Given the results of the microCT and histological assays, it is likely that bone formation was relatively high in AEM-housed mice for the first half of the study, prior to adaptation of their activity and behavior to the new enclosure. This would have increased the relative mineralizing surface and contributed to the increased trabecular bone mass we documented via microCT. However, over the latter half of the study, the anomalous loading declined and bone formation decreased. This is evidenced by the aforementioned decrease in tibia osteoblast surface and decline in serum markers of bone formation. It is important to remember that all of these assays represent the state of bone physiology at the study endpoint only, which limits our interpretation. A single Calcein label was administered in order to limit variability in experimental design as mice in the parallel spaceflight experiment could only be administered a single label prior to launch. In retrospect, double labeling should have been utilized, as it would have provided dynamic indices. This will certainly be considered in future ground-based studies.

There were increased levels of serum phosphorus in AEM-housed animals versus vivarium. Parathyroid hormone (PTH) is one of the primary regulators of both serum calcium and phosphorus homeostasis (Bergwitz and Juppner, 2010), producing increased calcium release from bone breakdown and increased phosphorus excretion via the kidney. The relatively higher phosphorus levels suggest a lower PTH level. There was not, however, a difference in serum calcium. Given the proposed biphasic bone formation response, it may be that serum phosphorus levels were simply lagging behind calcium in returning to baseline following an early increase in bone formation. Additional insight could be gained from longitudinal measurement of serum PTH levels in future studies.

We must also consider that the observed increase in trabecular mineralizing surface, and potentially bone formation, occurred as a result of the reduced activity of bone-resorbing osteoclasts in AEM-housed animals. Normal (i.e., higher) levels of osteoclast-mediated bone resorption in vivarium-housed mice could have removed portions of the Calcein label that were not resorbed in the AEM-housed mice. The presence of these labels, and their measured size, are necessary for the quantification of histomorphometric parameters. These complications highlight one of the inherent limitations of single label histomorphometry (Arnett and Henderson, 1998). Future studies of the AEM should make use of double label histomorphometry over smaller time frames in order to definitively resolve early versus late changes in bone formation.

Differences in cortical bone parameters between AEM- and vivarium-housed mice were modest compared to findings in the trabecular compartment. There were no changes in cortical structural parameters, such as cortical bone volume or cortical bone thickness, for AEM versus vivarium. However, there was significantly lower femur stiffness for AEM-housed mice, accounted for by lower mineral content of the femur diaphysis. Compared to baseline, AEM-housed mice had greater maximum calculated resistance to torsion and cortical thickness – differences not seen in vivarium-housed mice. However, none of these changes were substantial enough to result in functional outcomes in terms of increased bone mass or strength. It is important to note, however, that cortical bone is generally less sensitive to loading and unloading compared
Greater trabecular bone mass and trabecular mineralizing surface and reduced osteoclast surface are the most striking effects of AEM housing. To account for these findings, we propose that the structure of the AEM enclosure itself and the dynamics of group housing likely contribute to altered animal behavior and increased mechanical loading. The AEM has been designed in such a way that it will necessarily modify skeletal loading during normal activity. The wire mesh walls of the AEM module allow the animals to climb on the walls and roof, a behavior that is different from the standard “two dimensional” floor movement found in vivarium enclosures. This wire mesh is critical, however, for mechanisms that remove floating waste in microgravity (Brooks, 1981; Smith et al., 1987). In addition, the wire mesh provides an anchor point for mice during spaceflight, helping to reduce confounding effects of excessive stress on animal physiology (Sonnenfeld, 1999). It is also important to note that while the animals in the AEM are not crowded by NIH standards, they do have 60% less floor area per animal than the standard vivarium enclosures (130 vs. 81 cm²/mouse). The increased density of animals could have led to increased movement (running, jumping, fighting, climbing over other animals, etc.) that could contribute to increased loading and thus increased bone formation and accumulation of bone mass. In addition, it has been proposed that the interaction and companionship afforded by higher density group housing in the AEM may result in less stress for the animals when compared to standard vivarium housing (Morey-Holton et al., 2000). Although not measured here, the stress hormone cortisol and other glucocorticoids are potent activators of bone resorption in both humans and rodents (Gluer et al., 2007; Rehman and Lane, 2003). If glucocorticoid levels were lower in AEM versus vivarium, it may have contributed to the increase in bone mass that was observed. Measurement of stress hormone levels and adrenal mass would be worthwhile additions to future studies involving the AEM.

The record of spaceflight experiments utilizing animal models has yielded variable results with regards to the effects of housing environment on skeletal properties. Indeed, some studies demonstrated rather dramatic skeletal effects of housing type (Lafage-Proust et al., 1998; Morey and Baylink, 1978; Turner et al., 1979; Yagodovsky et al., 1976), while others saw relatively few changes (Bateman et al., 1998; Turner, 1995; Wronski et al., 1998). The results are so variable that one paper describes “arrested bone formation” (Spector et al., 1983), while another concludes there were “normal levels of [trabecular] bone mass and bone formation” (Wronski et al., 1998). Both the exaggerated and blunted effects of spaceflight on the rat skeleton may be an effect of the differing flight and ground control hardware housing conditions. The effects we observed are modest by comparison to the aforementioned studies, yet demonstrate a significant and important influence of the AEM housing environment that should be considered when planning future spaceflight experiments.

A recent study by Blotter and colleagues investigated the effects of ground-based housing of mice in the proposed “Mice in Space” (MIS) system (Blottner et al., 2009), which has not previously flown in space. In this study, investigators compared 25 days of housing in the MIS to individually ventilated cages. The investigators found no difference in bone structure or mineralization between the two groups. However, it is important to note the differences between the MIS and AEM enclosures, as well as variables related to experimental design. Importantly, the MIS system does not have the same wire mesh walls as the AEM. These walls provide an attachment point for the animals during spaceflight, helping to reduce animal stress in microgravity as well as during take off and landing. During ground-based use of the AEM, the mice are able to climb the walls and roof of the AEM. As mentioned before, this increase in animal activity and loading may have contributed to the observed increase in bone mass. It is also important to note difference in animal age (3-4 months in MIS vs. approximately 2 months in the present study), study duration (25 vs. 13 days), and animal sex (male vs. female) that complicate a direct comparison. The AEM has proven utility on multiple spaceflight missions, although additional investigation of the AEM and novel enclosure systems would be warranted before making a final decision on future use aboard the...
ISS or elsewhere.

A broad analysis of current literature performed by Morey-Holton and colleagues indicates that flight hardware housing parameters determined the degree to which spaceflight caused skeletal degradation (Morey-Holton et al., 2000). Specifically, they compared skeletal parameters from rats following nine days of spaceflight. Rats were housed in either AEMs (group housed with six rats in each AEM) or in the Research Animal Housing Facility (RAHF; singly housed rats in each habitat chamber). While individual, RAHF-housed animals experienced substantial declines in bone formation and bone mass, no significant changes were seen in AEM-housed animals. A similar study by Wronski and colleagues found that group housing of rats in AEMs essentially abolishes the effects of spaceflight on the skeletal system (Wronski et al., 1998). Although they did not use the AEM, other studies have demonstrated the confounding effects of group housing of animals on skeletal properties (Vico et al., 1993; Vico et al., 1988). We note that space-flown group housed rats are in relatively crowded conditions and experience substantial loading during normal social activities. It is for this reason that we proposed modifying the AEM to fly mice on STS-108 (Dalton et al., 2003).

Aside from rodent housing in spaceflight hardware, there is also precedent for the physiological effects of housing environment found in research regarding the bone structure of laying hens (Whitehead, 2004). As with rodent housing, the space available to hens and their ability to experience static and activity-associated loading correlated with positive bone mass and strength parameters (Newman and Leeson, 1998). For example, hens housed in cages with perches or in low-level aviary systems had greater leg bone strength, but relatively less of a change in wing bone strength, whereas birds permitted to fly in high-level aviaries showed relatively greater improvements in wing bone strength (Fleming et al., 1994; Knowles and Broom, 1990; Silversides et al., 2012). Positive effects of housing environment on bone quality in these animals are likely due to increases in osteoblast-mediated bone formation, rather than a decrease in bone resorption (Newman and Leeson, 1998; Whitehead, 2004). Much like the present study, this work demonstrates the complicated and perhaps unpredicted consequences of animal housing on bone.

There are many variables to consider when designing a ground-based control for the spaceflight environment, including the spatial distribution of animals, their range of movement, as well as the lack of buoyancy-driven convection. Conventional thinking would suggest that AEM-housed animals on the space shuttle or ISS should be compared to AEM-housed animals on the ground. However, the unique three-dimensional distribution of animals within the AEM during spaceflight, compared to their two-dimensional distribution on the ground, inevitably results in an imperfect comparison; however, this will be the case for any gravity versus microgravity comparison. Despite the limitations of the AEM as a ground-based enclosure, one cannot discount the impressive wealth of information that has been gleaned from spaceflight experiments, including those rodent studies that utilize the AEM. However, considering the complicated effects of the AEM on skeletal parameters presented here and in previous studies, and the logistical hurdles necessary for a complete redesign of flight hardware, it is clear that some adjustments are needed. The best recommendation would be to give the mice a longer acclimatization period to the AEM, or at least the wire mesh cage configuration. Giving the animals two weeks prior to the start of the flight experiment may help to negate some of the early behavioral, activity, and stress changes that occur as the mice adjust to their new environment. In addition, minimizing animal crowding, as much as possible, would be ideal.

In summary, the present study was conducted as a parallel ground-based control of a space shuttle flight experiment aboard STS-108. A comparison of mice housed in AEMs with mice housed in standard vivarium cages revealed a substantial influence of flight hardware on skeletal parameters. The effect of AEM housing was primarily evident in the trabecular bone compartment, with greater bone mass seen at the proximal tibia and humerus. These effects were likely due to a suppression of osteoclast-mediated bone resorption combined with an early increase in osteoblast activity. Although the specific reasons for this effect are not clear, it is reasonable to hypothesize that altered skeletal
loading, with mice living and climbing on the wire-meshed walls and interacting with each other, is a significant contributor. While the AEM will remain an integral part of animal experimentation on the International Space Station, it would be prudent to consider changes to both ground- and space-based utilization of these enclosures in order to minimize these effects.

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The Effects of Spaceflight on Mucin Production in the Mouse Uterus

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ABSTRACT

The effects of microgravity on biological tissues are relatively unexplored, especially in regard to the mammalian female reproductive system. To begin to address this issue, the uterine tissue of female mice flown on NASA shuttle mission STS-118 was studied. Three sets of female mice, each consisting of 12 animals, were utilized in this study: flight animals, ground control animals, and baseline animals. The flight animals were housed in the Animal Enclosure Module (AEM) of the Commercial Biomedical Testing Module-2 (CBMT-2), which was a part of the payload of the shuttle’s mid-deck locker. Ground control animals were housed in ground-based AEMs, which were kept in a room specifically designed to mimic the environmental conditions of the flight units with regard to temperature, humidity, and light/dark cycles on a 48 hour delay. Baseline animals were housed in standard rodent cages at ambient temperature and humidity and a 12/12 light/dark cycle. The uterine tissue was stained using an Alcian Blue Periodic Acid Schiff staining procedure and the apical mucin layer thickness was subsequently analyzed. Analysis of the mucin layer in the uterus revealed that the thickness of the mucin layer in the flight tissue was significantly thicker that the mucin layers of the ground control and baseline tissue.

INTRODUCTION

It is well documented that spaceflight, and simulated microgravity, have effects on many different tissues and systems of the body. Some of the non-reproductive systems investigated include skeletal muscle arterioles and regional blood flow (Arbeille et al., 1996; Delp, 1999), immune system (Armstrong et al., 1993; Chapes et al., 1993; Chapes et al., 1999) and skeletal system (Droppert, 1990; Milstead et al., 2004). Some of the reproductive tissues studied include Quail oviduct length (Skrobanek et al., 2008), Seminiferous Tubules (Kamiya et al., 2003; Motabagani, 2007; Forsman, 2012), and uterine smooth muscle (Burden et al., 1998). The vast majority of this information has been obtained by using animal models. One of the systems that has not been well studied is the female reproductive system. A report released in 1987 by the Space Studies Board and the National Research Council enunciated that it was particularly important to determine whether or not the space environment would interfere with human and/or animal reproduction (Moody and Golden, 2000). This was reiterated in the 2011 Decadal Study,

There have been studies to determine the effects of microgravity on development across different organismal taxonomies, such as sea urchins (Chakrabarti et al., 1995; Tash and Bracho, 1999), fish (Ijiri, 1998), Drosophila (Vernos et al., 1989), Xenopus (Smith and Neff, 1986; Huang and Johnson, 1995; Souza et al., 1995), quail (Huss et al., 2010), and rats (Wong and DeSantis, 1997). Although these studies have produced valuable yet sometimes conflicting data, they mainly focused on developmental studies and did not address the reproductive system as a whole. Additionally, from a human reproductive standpoint, it is imperative that data be obtained from vertebrate animals.

The opportunity to study the human female reproductive system in spaceflight has been relatively rare. The first woman to fly in space was Valentina Tereshkova. Her flight lasted approximately 2.9 days and no results of any tests on female reproductive/physiologic parameters that may have been conducted have been released. According to Schenker and Warmflash (online article, posted 2011), she later married a cosmonaut and a year later gave birth to a healthy girl. This became the world’s first evidence of a healthy post flight pregnancy. This further indicated that short term spaceflight did not appear to have any lasting effects on the reproductive tissues in either males or females. This conclusion was reinforced when American astronaut Margaret Rhea Seddon, who is married to former astronaut Robert L. Gibson, had an uneventful pregnancy and gave birth to a healthy baby after having been in space. Although this provides evidence that the human reproductive systems do not appear to suffer any long term effects from short term spaceflight, it provides little information about what changes may have occurred during the flight and whether or not any changes associated with long duration spaceflight would be reversible.

Unfortunately, there is still a lack of information regarding the human female reproductive system in spaceflight because female astronauts suppress their menstrual cycles during spaceflight (Jennings and Baker, 2000). This leaves scientists to rely on studies of animal models to extrapolate the possible effects on the human reproductive system. The few studies that have been conducted have mainly focused on embryo development and gestation. In 1979, male and female rats were allowed to comingle in an experiment flown on COSMOS 1129. The results indicated that none of the female rats gave birth; however, closer examination revealed that ovulation had taken place and that two of the rats had been pregnant, with subsequent reabsorption of the embryos (Serova and Denisova, 1982). Experiments conducted on pregnant rats that were flown on space shuttle from days 9-20 of pregnancy indicated that spaceflight did not affect placental structure (Renegar et al., 1995). Studies that evaluated the ovarian follicles, corpora lutea, luteinizing hormone, and follicle stimulating hormone from rats subjected to spaceflight following the post-implantation period found no effect of spaceflight on any of the parameters studied (Burden et al., 1995). In studies conducted by Burden et al. (1998) it was found that myometrial smooth muscle decreased by 37% between the 20th day of gestation and postpartum, compared to synchronous controls. Additionally, Burden et al. (1999) and Ronca and Alberts (2000) reported more numerous labor contractions in rats flown in space but delivering immediately post flight than that seen in control animals. Smith and Forsman (2012) evaluated the ovaries from non-pregnant mice from shuttle mission STS-118 and found no gross abnormalities. This is in contrast to the findings of Tash et al. (2011), who found that most follicles from spaceflight ovaries were atretic and that corpora lutea appeared in fewer numbers. They also found a trend toward smaller uteri in flight animals. Smith and Forsman (2012) evaluated the ovaries from non-pregnant mice from shuttle mission STS-118 and found no gross abnormalities. This is in contrast to the findings of Tash et al. (2011), who found that most follicles from spaceflight ovaries were atretic and that corpora lutea appeared in fewer numbers. They also found a trend toward smaller uteri in flight animals. Smith and Forsman (2012) provided a brief review of the effects of spaceflight as well as ground-based models on the male and female reproductive systems. The review reiterated that relatively little is known about the effects of spaceflight or ground-based models on the female reproductive system.

The uterine horn of the mouse has a uterine cavity lined with a specialized epithelium known as endometrium. As with most luminal surfaces within animals, the endometrium is coated with mucin. Mucins are glycoproteins that contain large numbers of O-linked oligosaccharides. Mucin is believed to provide lubrication, protection from pathogens, and also help prevent...
desiccation and enzymatic degradation. Mucins are located on the apical surface of many non-keratinizing stratified squamous epithelia such as uterine tissue (Gipson et al., 1995). Within the female reproductive system these mucins also provide a suitable environment for continued sperm maturation, gamete interaction, and early embryonic development (Gandolfi et al., 1989).

Eight varieties of mucins have been described in the reproductive tract of humans. These are denoted as MUC1 – MUC7 (with subsets MUC5AC and MUC5B). All reproductive tract epithelia express MUC1, which is not surprising since this transmembrane mucin is expressed by most epithelia (Warren and Spicer, 1961). Some mucins contain sialic acid and are generally referred to as a sialomucin complex (SMC). Muc4/SMC is abundantly expressed at the apical surfaces of most epithelia of the female reproductive tract, including both uterine luminal and glandular epithelia. Sialomucins can block cell and molecular recognition processes (Carraway et al., 1992) that renders the apical surface of cells with this type of mucin non-adhesive. Muc4/SMC is hormonally regulated in uterine luminal epithelia, but not in uterine glandular epithelium, oviduct, cervical, or vaginal epithelia (Idris and Carraway, 1999). It has further been reported that SMC expression is tightly regulated in the uterus, and its expression appears to block blastocyst implantation (McNeer et al., 1998; Carraway and Idris, 2001).

With the hypothesis that the spaceflight environment may alter mucin production in uterine tissue, this preliminary experiment used Alcian Blue Periodic Acid Schiff staining to determine the general pH range of the mucin in the uterus. Measurements of the thickness of the apical mucin layer were also conducted.

MATERIALS AND METHODS

The animals used in this study were a subset of animals utilized by the Amgen Corporation (Thousand Oak, CA). All mice used in these experiments were C57BL/6 female mice (Charles River, Wilmington, MA). The mice were initially divided into two groups of animals designated as drug treated mice (DM) and vehicle mice (VM). These groups were then subdivided into three treatment groups: flight (FL), ground control (GC), and baseline (BL). The drug treated group was proprietary and all tissues from this group were retained by Amgen. For all three treatment groups the VM were randomly mixed with the DM. All of the FL and GC mice were housed in the animal enclosure module (AEM) of the Commercial Biomedical Testing Module-2 (CBTM-2). The FL AEMs were flown on shuttle mission STS-118 in the shuttle mid-deck locker. This exposed the FL mice to approximately 13 days of spaceflight. The GC AEMs (housed at the Space Life Sciences Lab at Kennedy Space Center) were populated with the same number of mice as the FL AEMs and were conducted at a 48 hour delay from the FL animals to allow for reproducing the environmental conditions experienced on board the shuttle. Each AEM contained 8 mice configured 4 to a side. There were three FL AEMs for a total of 24 FL mice; 12 proprietary DM, and 12 VM that were available for this study. Accordingly, there were three GC AEMs for a total of 24 GC mice; 12 proprietary DM, and 12 VM that were available for this study. The BL mice were housed in standard rodent cages at the same population density. These mice were also housed at the Space Life Sciences Lab at Kennedy Space Center. The 12 BL VM were available for this study. All mice were approximately 9 weeks old at the onset of the mission. Upon mission completion, the reproductive tissues were harvested from each animal, fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded using standard embedding techniques. The embedded tissue was stored until use in this study.

Tissues were sectioned at 4 μm on a Microm HM325 microtome, mounted on glass microscope slides, and stained using an Alcian Blue Periodic Acid Schiff staining technique. The tissue was then examined and photographed using a Zeiss Axiostar 40 microscope equipped with a Canon Powershot A640 camera. For each animal sample, three slides were prepared. The stained tissue was analyzed for the type of mucin present and the thickness of the mucin layer. The FL, BL, and GC tissue was quantitatively analyzed, comparing mucin thickness between the three groups. Using a randomization grid, a set of five random measurements of the thickness of the mucin layer was made for one random section of tissue on each slide, giving a total of 15 measurements per sample. Measurements were made using the Carl
Zeiss AxioVision software, version 4.7.0. The average mucin thickness was then calculated for each sample. A one-way ANOVA was conducted using MiniTab statistical software.

RESULTS

Analysis of the Alcian Blue Periodic Acid Shiff stained tissue revealed good color differentiation of the apical mucin layer. Using this technique acidic mucins will stain a pale blue, neutral mucins will stain magenta, and mixtures of acidic and neutral mucins will stain purple. Qualitative evaluation of the color of the mucin layer between the three treatment groups did not indicate a consistent mucin type, based on general pH range.

Quantitative analysis of the thickness of the mucin layer did reveal significant differences between the FL tissue and the two control tissues. The values indicated are the mean values of the mucin thickness from each animal. Statistical analysis using one way ANOVA indicated that the mucin layer of the FL tissue was significantly thicker than that of the BL tissue (P=0.003, n=20) as well as the GC tissue (P=0.003, n=19), but that the thickness of the mucin layer in the BL and GC tissue was essentially the same thickness (P=0.922, n=17). Graph 1 clearly indicates the similarity between the two control groups as well as the increased thickness of the mucin layer in the flight tissue.

The mucin layer of the uterine tissue from FL animals maintained a relatively uniform thickness throughout the folds of the uterine wall. This thickness is indicated in Figure 1, which also indicates that this particular tissue had a mixture of acid and neutral mucins, based on the purple color of the stain.

The mucin layer of the BL tissue was relatively thin across all folds of the uterus. Figure 2 indicates a mixture of acidic and neutral mucins in this particular sample as indicated by the purple staining of the mucin. Some variation in the thickness of the mucin layer can also be seen.

Analysis of the GC tissue indicates a neutral mucin layer in this particular animal as can be seen in Figure 3, as indicated by a pale blue staining of the mucin. A slight variance in the mucin thickness can also be seen.

Graph 1. Comparison of the Mean mucin layer thickness from each animal across all treatment groups.
Figure 1. Uterine tissue from a FL animal. Note the relatively uniform mucin thickness throughout the folds of the uterine wall as well as the mixture of acid and neutral mucin as indicated by the purple staining (arrows) 400X.

Figure 2. Uterine tissue from a BL animal. Note the relatively thin mucin layer across all folds of the uterus as well as the mixture of acidic and neutral mucin as indicated by the purple staining (arrows) 400X.

Figure 3. Uterine tissue from a GC animal. Note the neutral pH of the mucin as indicated by the pale blue staining (arrows) as well as a slight variance in the mucin thickness throughout the uterine folds (400X).
DISCUSSION

The results of the Alcian Blue Periodic Acid Schiff staining were inconclusive. There appeared to be no correlation between the general acidity of the mucin and treatment. Some, but not all of FL mucin stained purple, indicating a mixture of acidic and neutral mucins. The same observation was made in the GC tissue. Although the GC mucin generally stained a pale blue, indicating a neutral pH, some GC tissue exhibited a purple staining. Very few of the tissues from any of the treatment groups exhibited magenta staining, which would be indicative of an acid mucin.

The most obvious explanation for the variation in mucin pH within a treatment group would be that the various animals were in different stages of their estrous cycle at the time of sacrifice. As previously stated, the mucin layer in the uterus is the only mucin of the female reproductive tract that is sensitive to hormonal changes. Because this was a tissue sharing experiment, we did not have access to blood for hormone sampling, nor were we able to conduct vaginal smears to determine the stage of the estrous cycle for each animal. Due to the nature/design of the AEM and the overall flight experiment, it is reasonable to assume that the Lee-Boot effect (Whitten, 1959) arrested all of the animals in an extended state of diestrous. If that were the case, we cannot ascribe these findings to varying stages of the estrous cycle. This variation may also be a function of the specific region of the uterus sampled. This cannot be confirmed because random uterine sampling was used in this study. From this data we must report that the spaceflight environment does not appear to have an effect on the type of mucin produced in regard to pH, and we attribute these findings to individual variation between animals.

Although there was a range of the mean mucin thickness in each of the treatment groups, statistical analysis clearly indicates that the apical mucin layer of the FL tissue was thicker than that of either of the control tissues. The same analysis indicates that the apical mucin layer in the control tissues (GC, BL) did not differ in thickness.

With regard to the thickness of the apical mucin layer, clearly some aspect of spaceflight caused a thickening of that layer. Previous reports regarding the regulation of the mucin layer in the uterus of rats and mice support the findings of this study. Muc1 expression is high in the proestrous and estrous stages, stages that correspond with high estrogen levels, and is the lowest in the metestrous and diestrous stages, which are associated with high levels of progesterone (Surveyor et al., 1995; Idris and Carraway, 1999). This suggests that the loss of Muc1 contributes to a receptive uterine state (Surveyor et al., 1995) at the time of implantation. If the animals in this study were arrested in an extended diestrous, we would expect to see a thin apical layer of mucin in the uterus, across all treatment groups. Since the apical mucin layer was significantly thicker in flight animals, this would suggest that the spaceflight environment causes a thickening of the apical mucin layer in the uterus. This would result in two important outcomes: 1) the thicker mucin layer would afford the uterus with greater protection from microbial invasion, and 2) there would be a greater barrier to blastocyst implantation (McNeer et al., 1998).

The aspect of spaceflight that would allow for the thickening of the uterine apical mucin layer is yet to be elucidated. One possibility would concern the pituitary gonadal axis. Spaceflight has been shown to cause changes in the anterior pituitary gland (Pattison et al., 1991). If these changes interfere with the production of follicle stimulating hormone (FSH) or luteinizing hormone (LH), they may lead to changes in mucin production in the reproductive tissues. Experiments involving pregnant rats that were flown on space shuttle Atlantis indicated that spaceflight had no effect on plasma progesterone concentrations or plasma LH concentrations but did significantly increase plasma FSH levels (Burden et al., 1995; Burden et al., 1997). The same studies showed a significant decrease in pituitary content of LH but no apparent effect on the pituitary content of FSH.

Another factor that must be considered when evaluating spaceflight tissue is the possible effect of cosmic radiation. During spaceflight astronauts and test animals are exposed to high levels of, and sometimes prolonged exposure to, cosmic radiation. This high energy radiation has an ionizing effect on the atoms and molecules of the biological system (Setlow, 2003). Ideally, GC conditions would allow for irradiation of the
experimental animals to mimic the radiation levels experienced by the flight animals. At this point in time that control is not practical.

In summary, to date this has been the only study of the effects of spaceflight on the mucin layer of the mouse uterus. This study indicates that the spaceflight environment causes a thickening in the apical mucin layer of the mouse uterine epithelium. There were several limitations to this study. These findings provide data that supports reinvestigating this area. A new study should be conducted that will allow investigators to ensure that the Lee-Boot Effect is not a factor in the study. Along these lines investigators should have access to blood for hormonal analysis as well as vaginal smears. Both of these would allow for accurate assignment of the stage of the estrous cycle for each animal in the study. For completeness of study there should be a mechanism to irradiate the GC animals with doses of radiation similar to those experienced by the flight animals. Future studies may also investigate changes in gene expression of the mucin producing cells.

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Spaceflight Effects and Molecular Responses in the Mouse Eye: Preliminary Observations After Shuttle Mission STS-133

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ABSTRACT

Spaceflight exploration presents environmental stressors including microgravity-induced cephalad fluid shift and radiation exposure. Ocular changes leading to visual impairment in astronauts are of occupational health relevance. The effect of this complex environment on ocular morphology and function is poorly understood. Female 10-12 week-old BALB/cJ mice were assigned to a flight (FLT) group flown on shuttle mission STS-133, Animal Enclosure Module ground control group (AEM), or vivarium-housed (VIV) ground controls. Eyes were collected at 1, 5, and 7 days after landing and were fixed for histological sectioning. The contralateral eye was used for gene expression profiling by RT-qPCR. Sections were visualized by hematoxylin/eosin stain and processed for 8-hydroxy-2'-deoxyguanosine (8-OHdG), caspase-3, and glial fibrillary acidic protein (GFAP) and β-amyloid double-staining. 8-OHdG and caspase-3 immunoreactivity was increased in the retina in FLT samples at return from flight (R+1) compared to ground controls, and decreased at day 7 (R+7). β-amyloid was seen in the nerve fibers at the post-laminar region of the optic nerve in the flight samples (R+7). Expression of oxidative and cellular stress response genes was upregulated in the retina of FLT samples upon landing, followed by lower levels by R+7. These results suggest that reversible molecular damage occurs in the retina of mice exposed to spaceflight and that protective cellular pathways are induced in the retina and optic nerve in response to these changes.

INTRODUCTION

The space environment creates challenges for extended human spaceflight and presents a unique combination of stressors: microgravity, high-energy-particle radiation, nutritional deficiencies, hypobaric hypoxia, intermittent hyperoxia, and psychological stress. Lack of gravity implies reduced physical loading, fluid shift, and incompletely understood cellular responses that are reflected by a number of detrimental changes, such as muscle atrophy and loss of bone mass, immunosuppression, and overall gene expression changes (Pietzsch et al., 2011; Sundaresan and Pellis, 2009). Ground models of simulated
microgravity, namely hindlimb suspension (HS) and bed rest, induce a fluid shift and concomitant vascular pressure and flow alterations (Hargens and Watenpaugh, 1996; Wilkerson et al., 2002), affecting not only cardiovascular physiology but also inducing genome-wide gene expression changes in the central nervous system (Frigeri et al., 2008).

Ocular changes have been reported related to exposure to the space environment. In humans, the direct effect of radiation in the lens results in cataract formation (Cucinotta et al., 2001), which manifests with a higher incidence and earlier onset in the astronaut population. Light flashes in the eye are an occurrence that has been observed by astronauts since the Apollo program (Sannita et al., 2006) -- a phenomenon not completely understood.

Most importantly, recent medical data from astronaut cohorts have reported the development of optic disc edema, choroidal folds, posterior globe flattening, and a resulting hyperopic shift (Kramer et al., 2012; Mader et al., 2011) in a fraction of the astronaut population upon return from missions longer than 30 days (NASA, 2010). No clear etiology has been established for these cases, but it is hypothesized that microgravity, the ensuing cephalad fluid shift, and venous congestion may play a role. The perturbations observed in some individuals of the astronaut cohort resemble those found in papilledema associated with idiopathic intracranial hypertension (IIH) also known as pseudotumor cerebri (Friedman, 2007; Kramer et al., 2012; Mader et al., 2011). Because the etiology is still a matter of speculation, investigating whether exposure to microgravity represents a source of stress for the eye is an issue of critical occupational health importance. To this aim, this project examines the effects of spaceflight on the rodent eye and the responses that occur when challenged with exposure to microgravity in combination with other stressors during spaceflight.

Previous spaceflight studies performed on rodents found evidence of retinal degeneration in neonatal rats aboard shuttle mission STS-72 (Tombran-Tink and Barnstable, 2006), and of cell swelling and disruption in rats aboard two experiments on Russian Cosmos satellites (Philpott et al., 1980; Philpott et al., 1978). However, these studies were limited to structural histopathologic observations of the eye. In the present work, we expand the immunohistopathologic analysis to investigate the effects of spaceflight and the elicited responses observed in the eyes of mice aboard shuttle mission STS-133, focusing, for the first time, on molecular and cellular processes subjacent to the histopathologic changes.

MATERIALS AND METHODS

Animals

This work consisted of a tissue sharing-derived project that used specimens collected from a parent animal experiment aboard shuttle mission STS-133. The original experiment included animals infected with respiratory syncytial virus immediately after return to Earth (study led by independent investigator Dr. Roberto Garofalo, from the University of Texas Medical Branch in Galveston). However, the work discussed in this article only included the non-infected control animals. Animal procedures were approved by the NASA Ames Research Center and Kennedy Space Center institutional animal care and use committees. The STS-133 mission occurred from February 24 to March 9, 2011, for a total duration of 12 days and 19 hours. Female 10 to 12 week-old BALB/cJ mice were assigned to one of three experimental groups: Flight (FLT), Animal Enclosure Module (AEM) ground controls, and vivarium-housed (VIV) ground controls. The flight animals (FLT) were housed in AEMs identical to the ground controls. The AEM is a self-contained habitat that provides ventilation, waste management, food, water, and controlled lighting (Naidu et al., 1995). It has previously been used in experiments studying rodent biology during spaceflight. The AEM flight unit is located in the middeck locker of the shuttle and its temperature is set at 3° to 8°C above the environmental middeck temperature. Lighting of 14 lux is set to a 12 hour day/12 hour night cycle. AEM ground controls were maintained in identical conditions at the Space Life Sciences Laboratory, Kennedy Space Center. Vivarium ground controls were housed in standard vivarium cages and conditions, on a 12-hour day/12-hour night light cycle at 200 to 215 lux. In view of the housing and lighting conditions
of the vivarium, the proper ground controls that allow measuring the effects attributed to spaceflight are the AEM-housed ground controls.

After sacrifice, one eye of each mouse from the three groups (FLT, AEM, and VIV) was collected at 1, 5, and 7 days after landing, and was fixed for histological examination. The contralateral eye was stored in RNALater and used for gene expression profiling by RT-qPCR.

**Materials**

The histological 4% paraformaldehyde-based fixative was obtained from Excalibur Pathology, Inc., Oklahoma City, OK. Goat polyclonal antibody to 8-hydroxy-2'-deoxyguanosine (8OHdG) (ab10802) and rabbit polyclonal antibody to activated caspase-3 (ab52181) were purchased from Abcam Inc., Cambridge, MA. Mouse monoclonal antibody to β-amyloid 1-16 was obtained from Millipore (Temecula, CA) and rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) was purchased from Dako, Carpinteria, CA. Paraffin embedding and histologic sectioning were contracted from Excalibur Pathology. qRT-PCR reagents were purchased from Qiagen Inc., Valencia, CA and BioRad, Hercules, CA. Tissue samples were assigned a different number for immunohistochemistry evaluation and gene profiling to perform a masked analysis.

**Histology and Immunohistochemistry**

Fixed eyes were paraffin embedded, sectioned at 5 µm thickness, and stained with standard hematoxylin-eosin (H&E) for histologic examination. Four immunohistologic stains were performed: 8OHdG to detect oxidative-related DNA damage, activated caspase-3 to study apoptosis, and double stain using β-amyloid as a marker of neuronal and axonal injury and GFAP as an indicator of glial activation. All immunostains had negative (omitting primary antibody) and positive (using known tissue that reacts with the antibody of interest) controls. For 8OHdG and caspase-3 staining, sections were equilibrated in water after deparaffinization and treated sequentially in 3% hydrogen peroxide, 1% acetic acid, and 2.5% serum (Vector Labs, Burlingame, CA) before incubating with the diluted primary antibody for either 2 hours at room temperature or overnight at 4ºC. After washing in phosphate buffer saline (PBS), the specimens were incubated with Vector ImmPress detection kit corresponding to the primary antibody’s host and counterstained with hematoxylin. For the double stain with β-amyloid and GFAP, antigen retrieval was performed with Dako target retrieval solution (a modified citrate buffer from Dako, Carpinteria, CA), steaming for 25 minutes, and then treated with peroxidase blocking buffer as above, and endogenous biotin blocked with Vector Avidin/Biotin blocking kit (Vector, Burlingame, CA). Staining for β-amyloid was done with the mouse-on-mouse peroxidase kit according to the manufacturer’s instructions (Vector Labs). Diaminobenzidine (DAB) was used for color labeling for β-amyloid (brown). For GFAP immunostaining, Dako’s streptavidin phosphatase kit was used with permanent red (red) as the chromophore.

**Qualitative Detection**

Morphology and histology were interpreted by an ophthalmic pathologist (masked for specific study groups) on H&E slides. Immunostained slides were evaluated for positivity of stain in a graded scale from 0 to 3+, where 0 indicated absence of staining and 3+ indicated marked positivity and more than 3 positive cells per layer. Immunoreactivity was evaluated in the corneal epithelium and endothelium, iris, lens, choroid, retinal ganglion cell (RGC) layer, inner nuclear layer (INL), outer nuclear layer (ONL), and optic nerve.

**Quantitative Detection**

To quantify oxidative-related DNA damage in the retina, densitometric quantification of 8OHdG immunohistochemistry was performed. Briefly, digital color images of the retina were processed using NIH ImageJ ver.1.68 (Abramoff et al., 2004) and converted to an 8-bit inverted grayscale image for analysis. Regions of interest were selected from each retina section, corresponding to the RGC, INL, and ONL as well as nearby areas without immunoreactivity for background measurements. Five sections were analyzed for each sample, for which the mean density per unit area (minus mean background density) was measured.

To quantify apoptosis in the retina, activated caspase-3 positive cells were identified for each
retinal sample and expressed over the total number of cells in each of the following retinal layers: RGC, INL, and ONL. Cellular number was determined with the cell counting plug-in for ImageJ ITCN (Byun et al., 2006).

**Gene Expression Analysis**

Mouse retina was microdissected and placed in RNAlater (Life Technologies, Grand Island, NY). Total RNA was then isolated using the AllPrep DNA/RNA Micro kit (Qiagen, Valencia, CA) and analyzed for quality using an Agilent 2100 Bioanalyzer. All samples used reported a RNA Integrity Number (RIN) >7.0. The Quantitect Reverse Transcriptase kit (Qiagen) was then used to generate cDNA templates for subsequent real-time qPCR analysis. Fifty nanograms of RNA were used in each reverse transcriptase reaction in a total reaction volume scaled to 30 μL according to manufacturer’s instructions, and the synthesis reaction was allowed to proceed for 2.5 hours. qPCR amplifications were done in a total volume of 20 μL using 1 μL of a 1:10 dilution of the cDNA pool obtained in the previous step and SYBR Green qPCR mastermix (BioRad, Hercules, CA) on a Bio-Rad CFX96 real-time PCR detection system. Samples were run in three technical replicates each. Primers (Qiagen) were selected to hybridize with genes specific for various cellular response pathways according to relevant findings in the literature that reported known roles in retinal stress, degeneration, oxidative stress, inflammation, and death/survival (Table 1). Three housekeeping genes (Hprt1, Rplp0, and Rpl13) were selected according to previously reported expression stability (van Wijngaarden et al., 2007). Normalization to the housekeeping genes was performed using the geNorm algorithm (Vandesompele et al., 2002) built into the CFX96 software, which computes a normalization factor for each sample from the contribution of each housekeeping gene.

**RESULTS**

**Histological Analysis of Eye Specimens**

Results are summarized in Table 2. All groups showed corneal acanthosis, defined as thickening of the epithelium of more than 5 layers of cells, and edema defined as clearing of cytoplasm with enlargement of the cell. However, irregular acanthosis, irregular increment of cell layers, with pronounced edema was present in the VIV group at R+7 (mice #41, 42). All mice had inflammatory cells either in the anterior chamber or vitreous, regardless of the group. Focal cortical cataracts, disrupted fibers, and formation of globules in the cortex of the lens, which is located between the nucleus and the epithelium, were present in several mice. As shown in Figure 1, full cortical cataracts were seen only in the two mice of the FLT group at R+7 group and this was associated with caspase-3 2+ staining. The VIV group at R+7 had no morphologic changes of cataract but had caspase-3 2+ staining as well (see below). Apoptosis of neurons defined as shrinkage of the cytoplasm with hyperchromatic nuclei and degenerated chromatin was observed in some mice. These findings were quantified using immunohistochemistry and they are discussed below. Some slides showed artifacts in the histology (possibly due to traumatic enucleation) that precluded complete interpretation. These findings are not included in the interpretation. Only those findings that are clear and not affected by processing are reported.

**Oxidative Stress: 8OHdG**

**Cornea**

8OHdG immunoreactivity was positive in all mice in the acanthotic areas of the cornea. In the FLT group, positivity was evidenced in the corneal epithelium and endothelium, but we were not able to document significant differences compared to AEM and VIV controls with the present data.

**Retina and Optic Nerve**

Figure 2 summarizes 8OHdG data. The two mice in the FLT group at R+1 showed frank positivity for 8OHdG in the neuronal layer. One of these also evidenced 8OHdG in some vessels over the ON head. Digital quantitative analysis of immunoreactivity in the retinal layers was more prominent in the RGC of FLT samples at R+1 (Figure 2B). Comparing FLT samples at the different tissue collection time points, 8OHdG immunoreactivity decreased from R+1 to R+7 (Figure 2B, C, D, and E). All mice were negative at the level of the optic nerve.
Table 1. Genes of interest evaluated for expression changes in the mouse retina. Grouping was done according to relevant cellular processes and complete gene name with gene symbol are provided, as well as references reporting possible relevant roles in retina physiology.

<table>
<thead>
<tr>
<th>Process</th>
<th>Gene Symbol</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death and survival</td>
<td>Bax</td>
<td>Bcl2-associated X protein</td>
</tr>
<tr>
<td>(Lohr et al., 2006)</td>
<td>Bcl2</td>
<td>B-cell lymphoma 2^1</td>
</tr>
<tr>
<td></td>
<td>Bag1</td>
<td>Bcl2-associated athanogene 1^2</td>
</tr>
<tr>
<td></td>
<td>Atg12</td>
<td>Autophagy related 12^3</td>
</tr>
<tr>
<td>Cellular Stress response</td>
<td>Hsf1</td>
<td>Heat shock transcription factor 1</td>
</tr>
<tr>
<td></td>
<td>Hspa1a</td>
<td>Heat shock 70kDa protein 1A^4</td>
</tr>
<tr>
<td></td>
<td>Sirt1</td>
<td>Sirtuin 1^5</td>
</tr>
<tr>
<td></td>
<td>Nfe2I2 (Nrf2)</td>
<td>Nuclear factor (erythroid-derived 2)-like 2^6</td>
</tr>
<tr>
<td>Oxidative stress response</td>
<td>Hmox1</td>
<td>Heme-oxygenase 1^7</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>Sod2</td>
<td>Superoxide dismutase 2, mitochondrial^8</td>
</tr>
<tr>
<td></td>
<td>Gpx4</td>
<td>Glutathione peroxidase 4^9</td>
</tr>
<tr>
<td></td>
<td>Prdx1</td>
<td>Peroxiredoxin 1</td>
</tr>
<tr>
<td></td>
<td>Cygb</td>
<td>Cytoglobin</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Nfkb1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1^10</td>
</tr>
<tr>
<td></td>
<td>Tgfb1</td>
<td>Transforming growth factor beta 1^11</td>
</tr>
<tr>
<td>Normalizing genes</td>
<td>Rpl13</td>
<td>Ribosomal protein L13</td>
</tr>
<tr>
<td></td>
<td>Rplp0</td>
<td>Ribosomal protein, large, P0</td>
</tr>
<tr>
<td></td>
<td>Hprt</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
</tr>
</tbody>
</table>

1 (Godley et al., 2002)  5 (Chen et al., 2009)  9 (Ueta et al., 2012)
2 (Liman et al., 2008)  6 (Wei et al., 2011)  10 (Wise et al., 2005)
3 (Wang et al., 2009)  7 (Zhu et al., 2007)  11 (Gerhardinger et al., 2009)
4 (Awasthi and Wagner, 2005)  8 (Justilien et al., 2007)

Apoptosis: Caspase-3

Cornea

Activated caspase-3 appeared positive in the cornea of all mice with the same intensity.

Lens

Two mice of the FLT group at R+7 had cataract formation associated with caspase-3 2+ staining (Figure 1). The VIV group at R+7 had no morphologic changes of cataract but had caspase-3 2+ staining as well.
cells than AEM samples at R+1, except for the INL in the AEM group at R+7. VIV samples also tended to increase their percentage of apoptotic cells at day R+7, as seen in qualitative analysis. Retinal pigment epithelium (RPE) of the FLT group at R+1 and one mouse at R+5 showed positivity with caspase-3, and one mouse AEM R+7 showed only rare and focal RPE staining (Figure 1). Qualitative and quantitative evaluation of ON immunoreactivity was inconclusive.

**β-amyloid and GFAP**

β-amyloid and GFAP stains were studied in the retina and optic nerve only and immunostained retina sections are shown in Figure 4. With regard to the retina, all mice were positive in the neuronal layer for β-amyloid. Overall, the vivarium mice showed a slightly higher positivity in both RGC and INL compared to the rest of the mice (VIV animals showed 2-3+ positivity at R+1 and R+5, more than any other group; one FLT animal at R+7 showed similar 2+ reactivity). GFAP was present in astrocytes of the retinal neuronal layer in at least one mouse of each group, except in the FLT group at R+5, where it was absent. No activation (positivity) of Muller cells was noted in any of the eyes.

While results were not conclusive from these retinal findings, it is important to note that only the FLT group at R+1 were positive for all stains at the retinal neuronal layer: 8OHdG, caspase-3, β-amyloid, and GFAP.

At the level of the optic nerve, only the FLT group at R+7 showed positivity for both β-amyloid in the axons and GAFP in the astrocytes either at the level of the lamina cribrosa or distal to it (Figure 4). No co-expression was seen of GFAP and β-amyloid in same cell type.

**Cellular Responses Identified by Gene Expression Analysis**

Gene expression profiling on STS-133 flight samples and their AEM and vivarium ground controls was performed targeting a set of genes focused on cellular death and survival, oxidative stress and cellular stress response, and inflammation. Results are shown in Figure 5 and Figure 6 and expressed as comparative normalized expression across the individual specimens at R+1 and R+7 for all groups. Due to the limited sample size, statistical analysis was not possible and these results are mainly descriptive.

**Activation of Oxidative Stress Response and Pro-Inflammatory Genes**

Figures 5 and 6 (see section below) plot gene expression data measured by real time qPCR. Several genes coding for key antioxidant enzymes (Hmox1, Sod2, Cat, Gpx4, Cygb, Prdx1) were elevated in retina samples obtained immediately after flight (Figure 5B), but this elevation returned

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**Table 2. Histologic interpretation with Hematoxylin-Eosin. Data arranged according to group (FLT, AEM, VIV) and day of sacrifice: 2 mice per group at R+1, +5, or +7, respectively.**

<table>
<thead>
<tr>
<th>Day</th>
<th>Cornea</th>
<th>Lens</th>
<th>Retina</th>
<th>ON</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLT</td>
<td>AEM</td>
<td>VIV</td>
<td>FLT</td>
</tr>
<tr>
<td>Day 1</td>
<td>FA and E</td>
<td>FA</td>
<td>FA</td>
<td>Anterior subcapsular C</td>
</tr>
<tr>
<td></td>
<td>Bullae*, A 1+, E 2+</td>
<td>Central E</td>
<td>A* 2+</td>
<td>FA</td>
</tr>
<tr>
<td>Day 5</td>
<td>FA and basal E</td>
<td>FA*, E 1+</td>
<td>Central E</td>
<td>FA</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>Intranuclear inclusions, A 1+, E 2+</td>
<td>FA</td>
<td>Nml</td>
</tr>
<tr>
<td>Day 7</td>
<td>FA</td>
<td>Irregular A 1+</td>
<td>E 3+</td>
<td>Cortical C</td>
</tr>
<tr>
<td></td>
<td>A* 1+, E 2+</td>
<td>Irregular A 1+</td>
<td>E 2+</td>
<td>Cortical C</td>
</tr>
</tbody>
</table>

(A)= acanthosis, (C)= cataract, (E)= edema, (FA)= focal acantosis, (Nml)=normal. anterior subcapsular C (anterior subcapsular cataract is disruption of the fibers with proliferation of the epithelium in the anterior subcapsular areas of the lens)

Comments: *Anterior chamber 1+ cell
to levels closer to AEM ground control values at 7 days post-landing. A similar trend was observed for inflammatory mediators Nfkb1 and Tgfb1 (Figure 5A).

Hmox1 showed the highest levels in those samples for which a higher evidence of stress was observed (FLT samples at R+1 and VIV ground controls).

Figure 1. Histological analysis of H&E and Caspase-3 stained eye samples. Hematoxylin and Eosin stain, original magnification 20X: Panel A. AEM R+7, Epithelium of cornea showing focal edema of cells seen as clearing and enlargement of the cytoplasm in the basal layers (star marks the level of the basal layers) and acanthosis (thickening of more than 5 layers of cells). Panel C. FLT R+1, anterior lens with cortical cataract seen as disorganization of the fibers of the cortex (arrows at the level of the cortex). Notice the displaced nucleus (nucleus of epithelial cells of the lens should only be present in the subcapsular area and not in the cortex in the anterior portion of the lens). Panel E. FLT R+1, retina with an apoptotic neuron seen as a shrunken cell with hyperchromatic condensed nucleus and eosinophilic cytoplasm (arrow head). Remainder of retina appears morphologically unremarkable. Caspase 3 immunostaining: Panel B. FLT R+1 corneal epithelium staining positively with Caspase 3 in the superficial layers and in the basal layers (star). Positive staining of the basal cells of the corneal epithelium is seen in the focal acanthotic areas, and in the upper differentiated layers (internal positive control). Panel D. FLT R+1 lens epithelium staining with Caspase 3; notice that cortex is negative. Panel F. FLT R+1, retina with caspase-3 staining of cytoplasm of neurons (*) predominantly with faint staining of the inner nuclear layer (inl) and inner segments of photoreceptors (pr). The cytoplasm of RPE cells is also staining (arrow).
Figure 2. 8OHdG immunoreactivity in retinal neuronal layers of AEM and FLT mice. Bars indicate the mean of n=2 biological samples. Each individual neuronal cell layer was compared at R+1, R+5, and R+7 in AEM samples (panel A) and Flight samples (panel B). Representative images of 8OHdG stained histological sections of the retina in FLT samples at R+1 (panel C), R+5 (panel D), and R+7 (panel E).
Figure 3. Quantification of Caspase-3 immunoreactivity by neuronal layer. Percentage of caspase-3 positive cells in the Inner Nuclear Layer (panel A) and the Retinal Ganglion Cell Layer (panel B) was calculated as described in Methods for day R+1 and R+7 tissue collection time points. Representative images of histological sections stain (red-brown) for caspase-3 of Flight (panel C), AEM (panel D), and Vivarium (panel E) samples at day R+1. Arrows indicate caspase-3 positive stained cells identified in different layers of the retina.
Figure 4. Beta amyloid (brown) and glial fibrillary acid protein (GFAP) (red) double staining immunohistochemistry. A: FLT R+1 (mouse #13). Retina with focal positive cytoplasmic staining in neurons of the ganglion cell layer (*) with β-amyloid (brown). Perivascular (arrow) and other astrocytes in the ganglion cell layer stain with GFAP (red). Notice the negative staining of Muller cells with GFAP. B: FLT R+1 optic nerve. Note the staining of the optic nerve (O.N.) in the region posterior to the lamina cribrosa (l.c.) with GFAP and focally with β-amyloid. Non-specific staining of the orbital muscle is also seen with β-amyloid (brown). C: FLT R+1 retina higher magnification of focal positivity with β-amyloid (brown) in ganglion cell layer (*) and GFAP in astrocytes (red). D: FLT R+1 optic nerve higher magnification of immediate post-laminar region. Notice the staining of oligodendrocytes and astrocytes with GFAP (red) and the β-amyloid stain (brown) of the nerve fibers in between the glial cells.

Cell Death and Survival Genes

The proapoptotic gene Bax was elevated in one flight sample (#13) at day R+1 and moderately elevated in one flight sample (#52) at R+7. Vivarium mice showed a higher expression of Bax at all collection time points compared to AEM ground controls. FLT samples at R+1 and VIV samples exhibited higher levels of the autophagy marker Atg12 and the survival genes Bcl2 and Bag1, suggesting that cellular protection mechanisms may be triggered as a response to cellular stress (Figure 6A).

Activation of Cellular Stress Genes

The cellular stress response genes Hsf1 and Nrf2 (Nfe2l2) were expressed slightly higher in VIV samples compared to AEM controls. Among the FLT mice, there was a tendency to higher expression at R+1 than R+7 (Figure 6B). The Hsf1 activator sirtuin 1 (Sirt1) did not show major differences across the various samples. Interestingly, the heat shock protein 70KDa Hsp1a1 was expressed at a lower level in mouse #13 that exhibited, overall, the highest signs of stress.
Figure 5. Gene expression analysis of inflammatory and oxidative stress response genes. Inflammatory response (panel A) and oxidative stress (panel B) gene expression levels from RNA isolated from retina samples in Flight (FLT), AEM, and Vivarium (VIV) samples at day R+1 and R+7, measured by real time qPCR. Y axis represents the comparative gene expression levels normalized to housekeeping genes.
Figure 6. Gene expression analysis of cell death and survival and cellular stress response genes. Cell death and survival (panel A) and cellular stress (panel B) gene expression levels from RNA isolated from retina samples in Flight (FLT), AEM, and Vivarium (VIV) samples at day R+1 and R+7, measured by real time qPCR. Y axis represents the comparative gene expression levels normalized to housekeeping genes.
DISCUSSION

While the spaceflight results reported herein represent pilot data due to the small sample size, these data offer, for the first time, direct evidence suggesting that oxidative stress, neuronal damage, and mechanical injury take place in the retina, lens, and optic nerve of rodents flown in low-Earth orbit for a period under two weeks. Several previous studies have shown the occurrence of oxidative stress during spaceflight (Stein, 2002), however, our work gives a first insight into the impact of space-associated factors on biological processes like cell death, oxidative stress, and probable mechanical injury in the rodent eye.

Because the BALB mouse strain used in the STS-133 experiment is susceptible to light-induced retinal degeneration (LaVail et al., 1987), we speculate that this particular strain exhibits an enhanced sensitivity to oxidative stress and/or a reduced stress response, making it a suitable strain in which to identify alerting evidence of risks previously unrecognized in the retinal tissue, while impacting its value as a model for the study of the human changes seen in-flight.

8OHdG, a product of deoxyguanosine oxidation, is a marker of oxidative stress-induced DNA damage. This damage has been observed in mouse cornea exposed to dryness (Nakamura et al., 2007), ultraviolet radiation (Tanito et al., 2003), and in mouse retina exposed to intense light (Tanito et al., 2002; Wiegand et al., 1983). In our study, 8OHdG was present in all acanthotic areas of the cornea. Irregular acanthosis with visible edema was only seen in the VIV samples at R+7, and it was only in this group where positivity at the corneal endothelium was observed since day 1, suggesting an impaired ion and water transport in the cornea.

The retinal response to intense light in susceptible mice has been studied before and has been found to be related to lipid peroxidation at the ONL (Tanito et al., 2002; Wiegand et al., 1983). Likewise, radiation-induced retinopathy is an ocular complication in cancer patients that receive radiation therapy (Parsons et al., 1996). The processes involved in the damage by high-energy-particle radiation in these cases may share commonalities (direct DNA damage and oxidative stress) with exposure to radiation present during spaceflight. The present work shows evidence of both oxidative stress-induced DNA damage in the neuronal layers of flight mice retinas and of an oxidative stress response induced at the gene expression level in these mice. Short-term responsiveness to DNA oxidation followed by DNA repair has been studied longitudinally in blood of trauma patients (Oldham et al., 2002), suggesting that the attenuated DNA damage observed after one week of return from flight may be the result of DNA repair.

Of note, the ground controls kept in the vivarium exhibited a comparable level of retinal oxidative stress to the samples from flight, especially at longer exposures (day R+7). This is likely due to the fact that the illumination conditions in a standard vivarium room are approximately 15-fold in light flux compared to the illumination of an AEM, even if both maintain a 12 hour light-12 hour dark cycle.

Caspase-3 is a pro-enzyme that is activated in the intrinsic apoptotic pathway in all mammals (D’Amelio et al., 2010). In this study, all mice showed positivity for caspase-3 at the level of the cornea. This may be explained by the fact that caspase-3 immunoreactivity in the stratified epithelium of the cornea serves as an internal positive control due to the natural differentiation process that the basal cells suffer towards cornification. Apoptosis can be triggered by oxidative stress, brain trauma, or ischemia. In a model of brain ischemia, the area of neuronal apoptosis has been identified not in the infarct region but in the surrounding area, where the oxygen tension is decreased, but not absent (Pulsinelli et al., 1982). The presence of activated caspase-3 is thus related to hypoxic environment and radiation exposure. In our study, the FLT group at R+1 showed higher positivity compared to the rest of the groups. This may be related to radiation and microgravity exposure during spaceflight. It is important to point out that the effect of high-energy-particle radiation may be overall increased in this susceptible mouse strain.

Qualitative examination revealed that VIV and FLT groups showed more caspase-3-positive cells at the retinal layers than AEM retinas. This may suggest that the damage caused by visible light radiation in the albino strain in the vivarium conditions may be comparable to the damage caused by the exposure to spaceflight.
environmental factors. We also observed positive microglial (astrocytes) but not Muller cell activation in VIV specimens, which may support the notion of visible light radiation effects as the triggering factor in inner layers of the retina only in these mice (Song et al., 2012).

Both mice in the FLT group at R+1 and one mouse at R+5 showed evidence of apoptosis in the RPE. Apoptosis in the RPE has been identified in ocular pathologies like age-related macular degeneration (AMD) secondary to exposure to activated monocytes (Yang et al., 2011), or triggered by oxidative stress with H\textsubscript{2}O\textsubscript{2}, lipofuscin, or light irradiation (Sparrow et al., 2000). This data also suggest oxidative stress may be an important component in the retinal damage in these mice. Of note, in vitro experiments with human RPE cells cultured in simulated microgravity generated by a NASA-bioreactor resulted in DNA damage and inflammatory response in these cells (Roberts et al., 2006). Retinal pigment epithelium attenuation has been related to retinal choroidal folds previously found in astronauts (Mader et al., 2011). It is yet to be determined whether or not increased RPE apoptosis may contribute to the formation of choroidal folds or if it increases the risk for AMD in astronauts.

Several advances in immunohistochemistry have led to the identification of \(\beta\)-amyloid in traumatic brain injury in humans (Iwata et al., 2002), rats, and pigs (Smith et al., 1999), by tracing not only the full-length protein but also small aminoacid peptides. \(\beta\)-amyloid was present in areas of the brain as soon as one day after brain trauma was provoked by pressure injection of saline into the cranium in a rat model (Pierce et al., 1996). Moreover, \(\beta\)-amyloid deposits showed evidence of optic nerve injury in cases of shaken-baby syndrome (Gleckman et al., 2000). Previous studies in animal models have shown distribution of \(\beta\)-amyloid in the mouse retina that suggests its involvement in the pathophysiology of glaucoma (Kipfer-Kauer et al., 2010). We report that \(\beta\)-amyloid deposition was present in the neural retina of mice in all treatment groups and that the VIV mice showed a slightly higher positivity in both RGC and INL compared to the rest of the mice. Interestingly, \(\beta\)-amyloid was present in the optic nerve of both mice in the FLT group at R+7 and had the unique characteristic of being at the level of lamina cribrosa or immediately distal to it. This compares with the findings in traumatic injury in children of shaken-baby syndrome where most of the axonal changes are seen in the postlaminar region (Gleckman et al., 2000). This may be associated to the anatomy of this region where the nerve is anchored by the fibers of the lamina cribrosa but immediately posterior to this or beyond this area the nerve can move freely. Thus, in the event of mechanical trauma the immediate fibers in the postlaminar region may be the ones demonstrating more damage. The trauma may include increased intracranial pressure that is transmitted into the nerve, positional or whiplash (similar, although in a less intense manner to what happens in shaken baby syndrome), or vibration (as the one occurring during launch or landing). However, there is the need to further investigate the nature of the changes through additional experimental work.

GFAP is an intermediate filament protein known to be present in astrocytes, Muller cells, and oligodendrocytes in the post-laminar optic nerve. GFAP is elevated when there is stress in the central nervous system and has been shown in the injured retina mostly present in the activated Muller cells (Lewis and Fisher, 2003). In this paper, we show that the optic nerves of several mice were positive for GFAP and \(\beta\)-amyloid; however, it was only the FLT group at R+7 that showed increased expression of GFAP at the postlaminar optic nerve. These findings suggest that the astrocytes and oligodendrocytes were activated in this region probable secondary to mechanical trauma. The causes of this, either vibration or fluid shift-related, need to be further investigated.

In addition, only FLT mice sacrificed at day 1 (FLT R+1) were immunoreactive in the neuronal layer for all \(\beta\)-amyloid, GFAP, caspase-3, and 8OHdG, suggesting increased oxidative and possibly mechanical damage. This may be explained by the possible correlation of \(\beta\)-amyloid deposition and activation of astrocytic cells, both triggering reactive oxygen species production (Lamoke et al., 2012).

The gene expression profiling results with BALB mice in flight STS-133 support the immunohistopathologic findings and suggest that:
These preliminary data suggest that spaceflight represents a source of environmental stress that directly translates into oxidative and cellular stress in the retina, which is partially reversible upon return to Earth. Moreover, the optic nerve findings suggest that the lesion may be mechanical in nature and that does not resolve after return to Earth, at least in the animals studied. Further work is needed to dissect the contribution of the various spaceflight factors (microgravity, radiation) and to evaluate the impact of the stress response on retinal and optic nerve health. These preliminary results should inform investigators on the design of future studies utilizing a more suitable mouse strain devoid of photic degeneration predisposition, male animals that better reflect the astronaut population, and statistically powered larger sample sizes.

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Effects of Underwater Arm-Cranking Exercise on Cardiac Autonomic Nervous Activity

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ABSTRACT

The purpose of this study was to clarify the beneficial effect of an underwater environment on heart rate (HR) and cardiac autonomic nervous activity (HF) during arm-cranking exercise. Ten healthy young men participated in this study. The arm-cranking exercise (40% peakVO₂) was performed for 10 minutes under two conditions: in water and in air. After the exercise, a recovery phase for 30 seconds followed. Changes in HR, VO₂, and HF did not differ between the conditions. The time constant of the heart rate decay for the first 30 seconds after exercise in the water was less than in air. The results suggest that cardiac parasympathetic nervous activity influences earlier recovery of HR after exercise in water. The results of our study suggest underwater exercise may be applied to wider areas of health management for individuals returning from space travel or sedentary patients in simulated microgravity environments.

Key words: Cardiac autonomic nervous system; underwater environment; heart rate; arm-cranking exercise; simulated microgravity; adaptation.

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INTRODUCTION

The human body’s autonomic nervous system controls important reflexes regulating heart rate and blood pressure. Space adaptation syndrome is one of the major complications associated with space travel (Thornton et al., 1987). This condition is not dependent on duration but occurs due to deconditioning of the circulation and nervous system. The pathophysiology is due to the decreased total body water, increased vein compliance, etc. These changes also occur among sedentary people, for example patients on prolonged bed rest, obesity, type 2 diabetes, etc. In Japan, it is believed that 22.1 million people are strongly suspected of having diabetes or presumed to have diabetes (Ministry of Health, Labour and Welfare, 2008). Many diabetic patients suffer from complications, such as diabetic nephropathy, retinopathy, and neuropathy. Epidemiological and intervention studies showed that endurance training was effective for improving the conditions of astronauts returning to Earth and aforementioned patients. There are various endurance trainings, namely treadmill walking, cycling, arm cranking exercise, etc. (Ono et al., 2005, 2006). In this study, we determined safe and effective exercise for such patients to avoid orthostatic hypotension. Hence, arm-cranking exercise was chosen. Exercise in air decreases renal blood flow depending on the intensity. However, renal blood flow is maintained during exercise in water. Moreover, buoyancy reduces loading on the leg joints. Additionally, prevention of deconditioning
due to spaceflight will lead to prevention of
deconditioning in sedentary patients.

The purpose of this study was to determine and
clarify beneficial effects of exercise underwater on
cardiac autonomic nervous activity during arm-

METHODS

Ten healthy young men (age: 21.7 ± 1.5 yrs,
stature: 171.0 ± 5.7 cm, body mass: 60.7 ± 3.8 kg,
BMI: 20.4 ± 1.7; mean ± SD) participated. This
study was granted approval by the Health Sciences
Ethics Committee in Kobe University.

Initially, the participants’ peakVO₂ was assessed
to set the intensity of arm-cranking exercise on land.
We used the arm ergometer (Monark; portable
ergometer 881E) in this study. Before assessment,
the height of the participants’ acromial process and
ergometer’s axis of rotation were set at the same level.
Arm-cranking exercise was performed with gradual
increase in the load every 2 minutes. Cranking was
done at 50 revolutions per minute (rpm). Examination
was stopped when revolution could not be maintained.
Cranking was conducted with mask on the
participants’ face in order to measure the VO₂ by gas
analysis (Arco System; AR-1). The participants
breathed through a mask connected to a flowmeter for
continuous measurement of inspired and expired
volume and by sensor for continuous measurement of
O₂ and CO₂ concentration. VO₂ was computed, at 15-
seconds interval, using the gas analysis software
(Arco System; AR-1 O-jiro series Ver.3.47) through a
personal computer (Toshiba; dynabook satellite T42).

The following day, examination was started with
a rest on land in a sitting position for 5 minutes. After
that, participants entered a water-filled tub and rested
in a sitting position for 10 minutes. After the rest in
the tub, they started arm-cranking exercise for 10
minutes. The participants exercised at 50 rpm
controlled by a metronome. The intensity of exercise
was equivalent to 40% peakVO₂. Two conditions
were set: exercise in the water (W-condition) and
exercise on the ground (C-condition) as control. All
of the experimental procedures were performed in
two randomized sessions on separate days at the
almost same time each day. The W-condition’s water
depth was at the level of the xiphoid process. After
the exercise, recovery was allowed for 30 seconds
continuously in the same position. The parameters
for measurements were oxygen uptake (VO₂), heart
rate (HR), rating of perceived exertion (RPE) and
rating of perceived fatigue sense (RPFS) of the upper
extremity by using the Borg scale (6-20), and cardiac
parasympathetic nervous activity (high frequency:
HF) during the experiment and time constant of the
heart rate decay for the first 30 seconds after exercise
(T30). We measured HR and R-R interval by using
the HR monitor (GMS; Active Tracer). We calculated
HF by using the analysis system (GMS;
MemCalc/Tarawa). Room temperature, humidity,
and water temperature during the experiments were
27.4 ± 1.0 °C, 81.4 ± 11.3 %, and 30.4 ± 0.3 °C in W-
condition, respectively. Room temperature and
humidity were 26.8 ± 1.5 °C and 74.0 ± 10.9 % in C-
condition, respectively.

DATA ANALYSIS AND STATISTICS

Cardiac parasympathetic nervous activity values
(HF) were analyzed with logarithmic transformation
to normalize the data (ln HF). Data were expressed as
mean ± SD except for RPE. RPE was expressed as
median ± SD.

Repeated-measures factorial ANOVA compared
changes between the conditions in VO₂, HR, and ln
HF, respectively. The paired Student’s t-test
compared differences between the conditions in HR
at rest phase and T30, respectively. The statistically
significant value was set to p<0.05. Effect sizes
(Cohen’s d-presented as “d”) was calculated for
exercise induced changes in HR and T30 with 0.2, 0.5,
and 0.8 representing small, medium, and large effect,
respectively (Mullineaux et al., 2001).

RESULTS

The peakVO₂ was 27.0 ± 3.9 ml/kg/min.

The intensity of exercise was set at 11.5 ± 4.9 W.
Figure 1 shows changes in oxygen uptake during the
experiment. The levels of VO₂ of W-condition and C-
condition during the end of the arm-cranking exercise
for 2 minutes were 15.6 ± 1.8 ml/kg/min and 14.4 ±
1.8 ml/kg/min, respectively. No significant
differences were found in the oxygen uptake.

Figure 2 shows changes in heart rate during arm-
cranking exercise for 10 minutes. HR of W-condition
at rest (62.0 ± 8.3 bpm) was significantly lower than
that of C-condition (74.3 ± 8.6 bpm) (p<0.05,
d=1.46). The changes in HR during exercise were not
significantly different in the two conditions, though HR at the end of exercise in W-condition (109.4 ± 14.6 bpm) was lower than C-condition (123.6 ± 19.6 bpm).

Figure 1. Changes in oxygen uptake during the experiment. There were no significant differences in the two conditions, exercise in the water (W-condition) and on the ground as control (C-condition), at the same phase.

The end of the exercise’s RPE was 11 ± 2.6 at W-condition and 13 ± 3.3 at C-condition. The intensity showed from ‘Fairly light’ to ‘Somewhat hard.’ The end of the exercise’s RPFS was 15 ± 1.0 at W-condition and 15 ± 2.2 at C-condition. The intensity showed ‘Hard.’ The changes in RPE and RPFS were not significantly different between W-condition and C-condition.

Figure 3 shows changes in ln HF during the experiment. Ln HF at rest was 7.03 ± 1.08 at W-condition and 5.96 ± 1.59 at C-condition. These results were not significantly different, though the W-condition’s ln HF was higher than that of C-condition. Ln HF at exercise phase of both conditions was lower than at rest. These results were not significantly different between W-condition and C-condition.

T30 at W-condition was 153.5 ± 42.5 and at C-condition was 254.6 ± 97.7. T30 at W-condition was significantly lower than that of C-condition (p<0.05, d=1.31).
DISCUSSION

The participants’ peak VO₂ levels were similar to the preceding study (Arakane et al., 2011). Hence, valid testing before the experiment was feasible.

The VO₂, HR, RPE, and RPFS were not significantly different in the two conditions during exercise. The water level in W-condition was kept below the participants’ upper extremities to match the C-condition’s exercise pattern. It is suggested that since water resistance was not affected, there was no difference in the exercise intensity based on the condition. It is reported that the cross sectional areas of the vena cava during 60% VO₂max arm cranking exercise in water decreased in 10 minutes and became equal to the areas on land exercise at 10 minutes (Onodera et al., 2005). Actually in this study, the intensity at the end of exercise showed about 59% peak VO₂ at W-condition and 54% peak VO₂ at C-condition. It is reported that arm cranking exercise’s peak VO₂ was about 60% of lower extremity ergometer exercise (Arakane et al., 2011). In addition to the increased HR during exercise in both conditions, a lower intensity than the calculated should be set when giving exercise prescription of arm-cranking in order to maximize the effect of underwater exercise. W-condition’s T30 was significantly lower than that of C-condition. The participants at W-condition may have been affected by water pressure that led to increased venous return during the recovery phase. T30 could be an index of vagally-mediated heart rate recovery after exercise (Imai et al., 1994). With these factors, it is suggested that cardiac parasympathetic nervous activity plays a role in the early recovery of HR immediately following exercise in water.

CONCLUSION

Arm-cranking exercise in the sitting position in water could lead to faster recovery of heart rate than on the ground. This exercise is deemed safe in consideration of the human’s circulation. The results of our study suggest underwater exercise may be applied to wider areas of health management for individuals returning from space travel or sedentary patients in simulated microgravity environments.

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REFERENCES


Development in Altered Gravity Influences Height in *Dictyostelium*

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**ABSTRACT**

We investigated the effects of altered gravity on the life cycle of *Dictyostelium discoideum* after and during life-long exposure to one of three altered gravity (g) environments: (1) substrate inverted, parallel to and facing the surface of the Earth; (2) hyper-g; (3) reduced-g. To this end, we measured the height of the final stage of the life cycle, the mature spore-bearing sorocarp. Typically, the sorocarp stands erect and perpendicular to the substrate. In the case of each altered g environment, the control cultures were produced and treated identically to the experimental cultures except for the conditions of their exposure to altered g. Inverted cultures developing and growing in the same direction as the gravity vector had a mean height of 1.84 mm. Their counterpart control cultures had a mean height of 1.64 mm being therefore statistically significantly shorter. Cultures chronically exposed to a hyper (10) g environment produced sorocarps with a mean height of 1.13 mm. These were statistically significantly shorter than their 1 g controls whose mean height was 2.06 mm. Clinorotated (simulated reduced g) sorocarp heights (mean equal to 2.12 mm) were statistically significantly taller compared to their 1 g controls (mean equal to 1.79 mm). The significance level for all the statistical analyses is p < 0.05. Therefore, measurements of the mature stage after life-long exposure to simulated altered gravity show that the final height of the sorocarp is ultimately determined, at least partially, by the gravity environment in which development occurs.

**INTRODUCTION**

Everything on Earth is subject to gravity. Since gravity on Earth is inaccurately considered a constant (1 g), its biological consequences are usually ignored. Human travel and colonization of space require knowledge of the effects of altered gravity on living systems as they transition from a nominal 1 g Earth environment to one of chronic altered (higher or lower) g. The clinostat is a ground-based instrument that attempts to simulate microgravity conditions (Albrecht-Buehler, 1992; Hader, 1999). Hyper g conditions can be simulated using a centrifuge designed to accommodate the organism under investigation (Benjaminson and Brown, 1988). In our lab, we have custom designed and fabricated instruments

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**Key words:** Hyper-g; Reduced-g; Clinostat; Sorocarp; *Dictyostelium*
that simulate both g conditions for non-human model systems (Benjaminson et al., 2006).

The Dictyostelium discoideum, Enterobacter aerogenes predator-prey system has proved to the authors to be a valuable tool for the study of the D. discoideum developmental cycle. D. discoideum has previously been used to explore the effects of gravity on development in both ground-based experiments (Kawasaki et al., 1990) and in space flight (Takahashi et al., 2001). Our experiments have revealed evidence that the rate at which Dictyostelium completes its life cycle is sensitive to changes in gravity (Benjaminson et al., 2006; Benjaminson, 1989a; Benjaminson, 1989b). Gravity has been shown to influence stalk height growth as well as germination efficiency, cell differentiation, and spore formation (Kawasaki et al., 1990). Here we report that the developmental morphology of Dictyostelium is also influenced by the gravity environment.

The transition from single celled amoeba, through a sequence of morphogenic changes, to multicellular spore-bearing reproductive sorocarp stage is well known to biologists (Athanasius and Hogeweg, 2001; Breen et al., 1992; Loomis, 1975). Under standard laboratory conditions, Dictyostelium sorocarps grow upward, away from the agar surface and opposite to the gravity vector. We had also observed, however, that cultures grown on agar surfaces in the inverted position with sorocarp erection in the same direction as the gravity vector, skip the migratory slug stage and tend to be taller than the upward facing and growing culminants (Benjaminson, unpublished data). Insights into the governance of size through the allocation of energy are helping us to answer important questions about the growth of individuals and the growth of populations (Kempes et al., 2012; McNab, 1984). The prospect of combining advanced energetic techniques and biotechnology in the search for knowledge of the balance among forces determining the fate of living things is an attractive one. Aside from the important role played by energetics, diffusing factors under genetic control determine size in Dictyostelium through governance of the number of cells in the multicellular stages (Brock and Gomer, 1999; Breen et al., 1992; Kamboj et al., 1990; Kopachik, 1982; Okuwa et al., 2001). Therefore, the interplay among genes, gene products, and gravity appears to have prescribed the final height of the sorocarps observed.

**MATERIALS AND METHODS**

Recognizing the probable variation in local environments inherent in our laboratory and in order to reduce the number of environmental variables, each individual experimental environment (inverted, centrifuged, clinorotated) housed its own controls. This provided both experimental subjects and their controls with essentially the same conditions.

**Dictyostelium Culture**

The D. discoideum strain used in these studies was the CB Wild Type stock, routinely fed on Enterobacter aerogenes bacteria (Carolina Biological, Burlington, NC 27215-3398). The experimental inocula were 24 hour co-cultures of CB amoebae and E. aerogenes in 0.1% Lactose-Peptone Broth (LPB), 0.1% lactose, 0.1% peptone in deionized water. One standard loopful (10 µl) of the mixed inoculum was spotted in the center of a 35X10 mm Lactose-Peptone Agar (LPA) plate. The Petri dishes were supplied by Falcon (Becton Dickinson, Franklin Lakes, NJ USA 07417) and the medium consisted of 0.1% lactose, 0.1% peptone, and 2% agar (all media components from BBL) dissolved in deionized water. All media were sterilized in an autoclave (Wisconsin Aluminum Foundry Co. Inc., WI) at 15 psi for 15 min. All plates were sealed with labeling tape to prevent desiccation and maintained in the dark at 25 degrees C throughout the tests to eliminate the effects of light on development (Benjaminson, in preparation).

**Inverted**

The first treatment involved merely inverting the experimental group of Petri dishes, orienting them with their lids down so that at culmination, the base of the sorocarp faced upwards away from the surface of the Earth and the spore-bearing sorophore hung downward.

**Hyper-g**

The second experimental condition involved exposure to hyper-g. The experimental cultures were subjected to 10 g, the same g value as those cultures centrifuged previously in Dr. Brown’s
Benjaminson et al. -- Altered Gravity Influences Dictyostelium Height

facility (Benjaminson and Brown, 1988). The Model D1006 centrifuge used in the current experiments was designed and fabricated by Lehrer Engineering, Pompton Lakes, New Jersey. This centrifuge was designed to provide a maximum hyper-g environment of 10 g. Precise rotational speed control and, hence, precise g values are obtained using a speed control with a feedback loop. The controls for this experiment were stacked on the floor of the centrifuge chamber. The centrifuge is shown in Figure 1.

Figure 1. Photograph of M1006 centrifuge showing housing and rotor.

Simulated Reduced-g

The reduced-g experimental group was rotated on a specially designed clinostat at 5.708 rpm, a speed calculated to simulate reduced-g (Benjaminson et al., 2006), Figure 2. The Model D1003 clinostat was designed and manufactured by Lehrer Engineering, Pompton Lakes, New Jersey. The reduced-g simulation is obtained by slowly rotating the clinostat disk holding the experimental cultures. Earth’s gravity vector with respect to the specimen is averaged out to approximately zero over each revolution of the disk. The rotational speed is sufficiently low (0.1 to 5.7 rpm) so that the effect of the centrifugal force induced by the rotation is essentially zero. The reduced gravity equals 0.0058 g at 5.7 rpm. The controls for this portion of the experiment were stowed in the clinostat’s upright U-shaped bracket alcove.

Figure 2. The D1003 clinostat viewed from above showing the rotor assembly with culture and the upright U-shaped bracket alcove.

Measurements

The mature sorocarps were prepared for measurement by removing the tape seals and placing a plastic cover slip (Thomas Scientific,
Swedesboro, NJ, USA 08085) on the exposed culture so that its weight forced the sorocarps into a supine position on the surface of the agar; see Figure 3. The length of the sorocarp from base-agar interface to distil terminus of the stalk where it joins the fruiting body was the dimension measured; see Figure 4.

![Figure 4. A rendition of a mature sorocarp showing the base, stalk, and fruiting body. The bracket delineates the measurement target.](image)

**Microscopy**

The microscope used was an Olympus IMT-2 inverted microscope (Olympus Corporation, Center Valley, PA 18034-0610) using the 2X objective. The 10X oculars were used for sighting. The randomly selected sorocarp images were captured and saved using a QICAM monochrome video camera equipped with QICAM operating software. Northern Eclipse software was used to measure the height of the sorocarps (all Empix Imaging, Inc., Mississauga, ON, Canada, L5L 5M6), and Excel software was employed for sorting and analyzing the data. Images were captured with the exposure time set at 16.4 ms. The Northern Eclipse measurement software was calibrated using a stage micrometer and the Petri dish was manually scanned. Images were loaded into Northern Eclipse software. Measurements were made using the tracing tool on the sorocarp image longitudinally (the tracing tool keeps track of the length of the trace in millimeters); the collected data were automatically saved to an Excel spreadsheet.

**RESULTS**

The three experiments were analyzed separately comparing the experimental group to its control group in each case. The independent student’s t test was used to compare the mean heights. Results can be seen in Table 1.

**Inverted**

In those *Dictyostelium* cultures that had simply been inverted, the sorocarps numbered 678. The average height of the sorocarps was 1.84 mm, ranging from 0.48 mm to 4.35 mm, a difference of 3.87 mm, with a standard deviation equal to 0.70 mm. The controls for this experiment were an average of 1.64 mm high and ranged in height from 0.44 mm to 3.89 mm, a difference of 3.45 mm, in the 642 measured, with a standard deviation equal to 0.66 mm.

For the inverted experiment, the calculated $t = 10.42$. For mean sorocarp height there is a significant difference between the inverted experimental group and the control group, $p < 0.05$.

**Hyper-g**

In the group centrifuged at 10 g, the number of sorocarps measured was 698, and they ranged in height from 0.50 mm to 5.01 mm, a difference of 4.51 mm, averaging 1.13 mm in length, with a standard deviation equal to 0.53 mm. The control for this group numbered 435 sorocarps. They ranged from 0.42 mm to 3.14 mm, a difference of 2.72 mm, with an average height of 2.06 mm, and a standard deviation equal to 0.75 mm.

For the hyper-g experiment, the calculated $t = 31.16$. For mean sorocarp height there is a significant difference between the hyper-g experimental group and the control group, $p < 0.05$. 
Table 1: Results.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Measurement</th>
<th>Inverted Experimental</th>
<th>Inverted Control</th>
<th>Hyper g Experimental</th>
<th>Hyper g Control</th>
<th>Simulated Reduced Gravity Experimental</th>
<th>Simulated Reduced Gravity Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of sorocarps (n)</td>
<td>678</td>
<td>642</td>
<td>698</td>
<td>435</td>
<td>1,121</td>
<td>1,353</td>
</tr>
<tr>
<td></td>
<td>Mean Height</td>
<td>1.84 mm</td>
<td>1.64 mm</td>
<td>1.13 mm</td>
<td>2.06 mm</td>
<td>2.12 mm</td>
<td>1.79 mm</td>
</tr>
<tr>
<td></td>
<td>+/- Standard Deviation</td>
<td>0.7 mm</td>
<td>0.66 mm</td>
<td>0.53 mm</td>
<td>0.75 mm</td>
<td>0.73 mm</td>
<td>0.68 mm</td>
</tr>
<tr>
<td></td>
<td>Range: High to Low</td>
<td>0.48 mm to 4.35 mm</td>
<td>0.44 mm to 3.89 mm</td>
<td>0.50 mm to 5.01 mm</td>
<td>0.42 mm to 3.14 mm</td>
<td>0.14 mm to 3.14 mm</td>
<td>0.37 mm to 5.07 mm</td>
</tr>
</tbody>
</table>

Simulated Reduced-g

In the case of the clinorotated cultures, 1,121 sorocarps were measured. The average height of the experimental group was 2.12 mm, ranging from 0.14 mm to 3.14 mm, a difference of 3.0 mm, with a standard deviation equal to 0.73 mm. The number of controls was 1,353 sorocarps, whose average height was 1.79 mm, ranging from 0.37 mm to 5.07 mm, a difference of 4.7 mm, with a standard deviation equal to 0.68 mm.

For the simulated reduced-g experiment, the calculated $t = 28.6$. For mean sorocarp height there is a significant difference between the simulated reduced-g experimental group and the control group, $p < 0.05$.

DISCUSSION

A number of gravity sensitive parameters have been previously identified in the life cycle (developmental pathway) of *Dictyostelium*. These included the rate of development and the rate of induction of germination (Benjaminson, 1997). Development is known to be governed by an array of gene products whose activity drives the life cycle forward (Loomis, 1975). Ultimately, the development of *Dictyostelium* is determined by the constraints imposed by its genes’ range of activity and the environment in which it develops. In the current study, we demonstrate that the final height of the sorocarp is dependent partially on the gravitational environment.

All the *Dictyostelium* amoebae employed in these experiments were derived from the same culture, and they all fed on the same population of bacteria under the same environmental conditions. Thus the control and experimental populations of *Dictyostelium* we used theoretically had access to equivalent amounts of stored energy partitioned for differentiation, movement, and biosynthesis (Benjaminson, 1997). To our knowledge the only experimental variable in this study was the gravitational environment.

The results of the experimental treatments vs. control treatments demonstrated that different gravitational environments had significant effects on sorocarp height each of the experimental groups being statistically significantly different in height from its control group. The inverted and simulated reduced gravity (clinorotated) groups had taller sorocarps as compared to their respective 1 g controls. The hyper-g experimentals (centrifuged) had shorter sorocarps as compared to their controls.

Inverted

In the “inverted” experiment, the control group was cultured with a typical orientation, i.e., the agar surface facing upward, resulting in *Dictyostelium* sorocarp assembly being vertical and perpendicular to the Earth’s surface (upright position). In this case, the gravity vector was in the opposite direction as compared to the sorocarp
assembly direction. For the experimental group, *Dictyostelium* was cultured in the inverted position, i.e., agar surface facing down. For the experimental group the gravity vector was in the same direction as the sorocarp assembly direction. Based on the results of this experiment, it appears that the gravitational environment of the experimental group somehow favored sorocarp assembly (erection) resulting in experimental (inverted) sorocarps being significantly shorter (p < 0.05) compared to controls. Based on our interpretation of graphs (Figures 5 and 6 in Kawasaki et al., 1990), similar results have been reported, in that the inverted fruiting bodies height (stalk height) was positively influenced (taller) by the inverted growing condition. We hypothesize that the inverted sorocarps received “help” from gravity for sorocarp assembly. This “help” may have been related to increased available energy consumption. Perhaps energy availability increased as a result of the elimination of the slug stage. Altered gene expression, epigenetic phenomena, other physical factors, and/or mechanical alterations may have played a role as well (Benjaminson, 1996).

**Hyper-g**

The hyper-g environment (10 g) produced shorter sorocarps when compared to 1 g controls. In the hyper-g experiment, the control group was housed on the centrifuge base in the upright position, both initiating and completing its life cycle in the 1 g environment. The 1 g gravity vector for the controls was in the opposite direction compared to the sorocarp assembly direction. For the experimental group, *Dictyostelium* was cultured in the upright position as well, i.e., agar surface facing upward. The gravity vector experienced by the experimental group was in the opposite direction as the sorocarp assembly direction, but exposed to 10 times (10 g) the force of Earth’s gravity (Benjaminson et al., 2006). Based on the results of this experiment, the gravitational environment of the experimental group negatively affected the sorocarp assembly resulting in experimental (centrifuged) sorocarps being significantly shorter (p < 0.05) compared to controls. This is in contrast to earlier experiments in hyper-g by Kawasaki et al. (1990) who reported that 3 g centrifugation throughout the entire *Dictyostelium* life cycle produced taller fruiting bodies (stalks) compared to 1 g controls. Our experiment used 10 g centrifugation on a different instrument platform and the number of stalks measured was different. In the measurement of the heights of fruiting bodies, Kawasaki reports that in the experiment, “thirty to 50 fruiting bodies were counted in each Petri dish, and three to 6 petri dishes measured.” That is, a maximum of 300 sorocarps were measured for height by Kawasaki’s group, compared to 678 (experimental) for a total of 1,121 in the experiments reported here. These differences may account for the conflicting results obtained.

We hypothesize that the hyper-g environment made sorocarp assembly more difficult, possibly requiring more energy expenditure and thereby resulting in shorter sorocarps than the 1 g controls.

**Simulated Reduced-g**

In the clinorotated experiment, the control group was housed on the clinostat chasis itself, completing its life cycle in the 1 g environment. The 1 g gravity vector for the controls was in the opposite direction compared to the sorocarp assembly direction. For the experimental group, the gravity vector theoretically approaches zero due to the *Dictyostelium* being attached to a substrate and going through 360 degrees of rotation during its entire life cycle, including sorocarp assembly. Theoretically, the clinostat speed set at 5,708 rpm partially cancels out the gravity vector through 360 degrees over time (Benjaminson et al., 2006). As with the inverted experimental group, the simulated reduced-g environment somehow favored sorocarp assembly, which resulted in experimental (clinorotated) sorocarps being significantly taller than their 1 g controls. This result is also in contrast to Kawasaki et al. (1990), who reported that chronic “artificial microgravity” produced by clinorotation, applied throughout the entire *Dictyostelium* life cycle, produced shorter fruiting bodies (stalks) compared to 1 g controls.

We hypothesize that the clinorotated sorocarps were taller due to the clinorotation reduced influence of a gravity vector acting in the opposite direction of sorocarp assembly and a reduced requirement for the expenditure of energy
to counterbalance gravity’s effect on the process of development.

**Hypothetical Mechanisms**

The allocation of energy for morphogenesis is governed by mechanisms that are sensitive to chronic simulated gravitational environments (Vasiev and Cornelis, 2003; Benjaminson, 1996; Benjaminson, 1997; McNab, 1984). Cell allocation may also have influenced morphogenesis as was described by Maeda and Maeda (1974), and reported by Kawasaki et al. (1990). These may account for the ordering of average heights of the experimental groups, ranging from longest to shortest, clinorotated to inverted to centrifuged.

**CONCLUSIONS**

The three experimental gravitational alterations produced measurable differences in the experimental groups. Our work, which expanded on Kawasaki et al. (1990) experiments, supports the hypothesis that gravity has an influence on morphogenesis in *D. discoideum*. Although our results differ from Kawasaki et al. (1990), the influence of gravity has been demonstrated in both experiments. How this influence is exerted and why results were different between our experiment and Kawasaki (Kawasaki et al., 1990) remains to be investigated.

**FUTURE EXPERIMENTS**

Based on the significant differences in *Dictyostelium discoideum* sorocarp heights observed in this study, we believe future investigations will confirm the results reported in this paper, and should extend to other model systems such as *Drosophila* and activated plant seeds. In addition, experiments designed to determine whether these effects are a universal phenomenon and to identify the underlying mechanisms of gravity’s influence on morphogenesis are planned.

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**Author Disclosure Statement**

No competing financial interests exist for ALL authors.

**REFERENCES**


A Computational Study of the Mechanics of Gravity-induced Torque on Cells

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ABSTRACT

In this paper we use Nace’s previous work in order to model the effects of gravity in cells and similar objects. In the presence of the gravitational field of a primary body, the gravity vector can result in numerous effects, some of which are tension, shear, and finally torque. To model the torque effect we use a complete expression for the gravitational acceleration, as this is given on the surface of a planetary body as well as in orbit around it. In particular, on the surface of the Earth the acceleration is corrected for the effect of oblateness and rotation. In the gravitational acceleration the effect of oblateness can be modeled with the inclusion of a term that contains the $J_2$ harmonic coefficient, as well as a term that depends on the square of angular velocity of the Earth. In orbit the acceleration of gravity at the point of the spacecraft is a function of the orbital elements and includes, only in our case, the $J_2$ harmonic since no Coriolis force is felt by the spacecraft. We derive analytical expressions and calculate the resulting torque effects for various geocentric latitudes, as well as circular and elliptical orbits of various eccentricities and inclinations. We find that elliptical polar orbits result in higher torques, and that higher eccentricities result in higher the torque effects. To any measurable extent, our results do not drastically impact any existing biophysical conclusions.

INTRODUCTION

Gravity is an important force that affects the life of a cell in various ways. When the gravitational force is exerted upon cells and similar objects, it results in cellular deformations and also in changes of the cell/object’s orientation. This may be due to compression caused by hydrostatic pressure that can affect the tensegrity of load bearing structures within cells, such as actin microfilaments and other cytoskeletal components (Vassy et al., 2001). In addition, it could be due to the influence it exerts on the physico-chemical self-organization of microtubules that comprise the cytoskeleton (Papaseit et al., 2000; Tabony et al., 2001). For example, Hughes-Fulford (2002) has demonstrated that serum deprived osteoblasts exhibit reduction in cell proliferation in...
microgravity conditions while these observations were confirmed by Vassy et al. (2001) in human breast cancer cells. In plant cells microgravity permits the enhancement of proliferation, perhaps due to reduced interactions in the absence of gravity (Matía et al., 2010).

The goal of this contribution is to reexamine the mechanics of gravity-induced torque in cells and similar objects, in surface and orbital experiments. For that we have considered three biological systems, i.e., sarcoma cells, human egg, and the Gallus gallus egg. The choice of these cells and similar objects is based on the fact that they vary increasingly in size. In addition, there is a scientific interest related to the gravitational effects of cancerous type of cells, along with the effect of gravitation at the basal stages of embryonic development. For that, our paper re-examines Nace’s result (Nace, 1983) that assumes a constant gravitational acceleration \( g \) and considers the gravitational acceleration on the surface of the Earth that is now corrected for geocentric latitude \( \phi_E \), the \( J_2 \) harmonic coefficient, and the rotation of the Earth via its angular velocity \( \omega_E \). These corrections are needed for accurate planet-based experiments. Similar calculations are repeated at the orbital altitude of an orbiting spacecraft, with the \( J_2 \) harmonic as the only correction included, accounting for the Earth’s oblateness. At the orbital altitude of the spacecraft the effect of the rotational potential of the Earth is not included since it does not affect the spacecraft, which is not in any physical contact with the Earth’s surface but is governed by the rotational potential of its orbit. Our calculations can be applied in the case where more accurate surface and orbital values of the gravitational potential are required. Our goal is to also derive and investigate the mathematical relations that relate biological experimental parameters to various Earth surface parameters as well as the orbital elements of the spacecraft. These planetary geophysical parameters include the \( J_2 \) harmonic, the flattening of the Earth \( f' \), and finally the angular velocity of the Earth’s rotation \( \omega_E \). Furthermore, the idea of using biological space experiments as an alternative method of calculating important planetary parameters in more advanced future experiments to come is also introduced.

**TORQUE ON CELLS AND SIMILAR OBJECTS**

In our effort to calculate the effect of gravity on cells and similar objects let us consider the gravitational potential \( V(r') \) at the surface of the Earth, where an experiment with cells and similar objects is taking place under controlled conditions. We can write the total potential as the sum of three different potential terms, namely:

\[
V_{\text{tot}}(r') = - \frac{GM_E}{r'} + \frac{GM_E R_E^2 J_2}{2 r'^3} \left( 3 \sin^2 \phi_E - 1 \right) - \frac{1}{2} \omega_E^2 r'^2 \cos^2 \phi_E ,
\]

where the first term in the right-hand side (RHS) represents the central Newtonian potential, the second term represents the potential correction due to the \( J_2 \) spherical harmonic of the Earth’s gravitational potential, and the third term represents the rotational potential, acting only on the surface of the Earth. Following Haranas et al. (2012), we write the magnitude of the total of all accelerations on the surface of the Earth to be:

\[
g_{\text{tot}} = \frac{GM_E}{r'^2} - \frac{3GM_E R_E^2 J_2}{2 r'^4} \left( 3 \sin^2 \phi_E - 1 \right) - \omega_E^2 r'^2 \cos^2 \phi_E ,
\]

where \( r' \) is the radial distance from the center of the Earth to an external surface point, \( M_E \) is the mass of the Earth, \( R_E \) is the radius of the Earth, \( J_2 \) is the zonal harmonic coefficient that describes the oblateness of the Earth, \( \omega_E \) the angular velocity of the Earth, and \( \phi_E \) is the geocentric latitude of the designed experiment. Zonal harmonics are simply bands of latitude, whose boundaries are the roots of a Legendre polynomial. This particular gravitational harmonic
coefficient is a result of the Earth’s shape and is about 1000 times larger than the next harmonic coefficient $J_3$ and its value is equal to $J_2 = -0.0010826260$ (Kaula, 2000). At the orbital point of the spacecraft the rotational potential on the surface of the Earth does not affect the orbit of spacecraft. Therefore the gravitational acceleration as it is given by Eq. (2) can be transformed as a function of orbital elements, using standard transformations given by Kaula (2000) and Vallado and McClain (2007), namely

$$\sin \phi \sin i \sin (u - \omega) \cos \theta,$$

where $\phi_E$ is the geocentric latitude, measured from the Earth’s equator to the poles, and $\theta_E$ is the corresponding colatitude measured from the poles down to the equator ($\theta_E = 90 - \phi_E$). $u = \omega + f$ is the argument of latitude that defines the position of a body moving along a Kepler orbit, $i$ its orbital inclination, $\omega$ is the argument of the perigee of the spacecraft (not to be confused with angular velocity, which we write with subscripts – see nomenclature section below), $f$ is its true anomaly (an angle defined between the orbital position of the spacecraft and its perigee). The orbital elements are shown in Figure 1 below.

![Figure 1. Explanation of the orbital elements: inclination $i$, argument of latitude $u = \omega + f$, and the radial vector $r'$ of the spacecraft, and $\lambda = 0$ is the zero longitude point on the Earth’s equator, and $x_\omega, y_\omega, z_\omega$ define a right handed coordinate system.](image)

To familiarize the reader with the orbital elements used here, let us define the orbital elements appearing in our Eq. (4) below. $a_s$ is the semi-major axis that defines the size of the orbital ellipse. It is the distance from the center of the ellipse to an apsis, i.e., the point where the radius vector is maximum or minimum (i.e., apogee and perigee points). Similarly, $e$ is the eccentricity, which defines the shape of the orbital ellipse (minor to major axis ratio), $i$ is the inclination of the orbit defined as the angle between the orbital and equatorial planes, and $\omega$ is the argument of the perigee, the angle between the direction of the ascending node (the point on the equatorial plane at which the satellite crosses from south to north) and the direction of the perigee. Finally, the true anomaly $f$ is the angle that locates the satellite in the orbital ellipse and is measured in the direction of motion from the perigee to the position vector of the satellite. Assuming an elliptical orbit, the geocentric orbital distance $r'$ is given by (Vallado and McClain, 2007):

$$r' = a_s \left(1 - e^2\right) \left(1 + e \cos f\right), \quad (3),$$

6 and therefore Eq. (2) becomes a function of the spacecraft orbital elements and therefore we have:
In relation to the torque we say that torque is defined as the moment of a force that measures the tendency of a particular force to produce rotation about an axis, and is defined according to the formula below (Meirovitch, 1998):

\[
\vec{L} = \vec{r} \times \vec{F} = Fr \sin \theta \tag{5}
\]

Figure 2. Nace’s floating cell used in this study to illustrate examples of torque. \(H\) and \(L\) are the heavy and light masses of the cell. \(r\) is the radius of the heavy mass and \(l_0\) is the total length of the cell, and \(\ell\) is the distance between the light and the heavy masses; \(d_H\) and \(d_L\) are the distance from these centers respectively, to the center of mass \(c.m.\) of the cell. \(V_H\) and \(V_L\) are the volumes of the heavy and light masses, and \(V_{BH}\) and \(V_{BL}\) are the volumes of the buoyant spheres surrounding \(V_H\) and \(V_L\). \(F_H\) and \(F_L\) are the force of gravity, and \(F_{BH}\) and \(F_{BL}\) are the buoyant forces respectively, acting on \(H\) and \(L\). \(g\) shows the direction of gravity, and \(\theta\) is the direction between \(F\) and \(\ell\).
where $F$ is the applied force, $r$ is the distance from
the center of mass that the force is applied to the
axis of rotation, and $\theta$ is the angle between $F$ and
$\ell$ is the distance between the centers of the heavy
and light masses in the cell, $\ell_0$ is the total cell length,
and $r_H = b r_{BH}$ where, where $r_H$ is the radius of the heavy mass and $r_{BH}$
the radius of the cell, $\rho_m$ is the density of the
medium, $\rho_H$ and $\rho_L$ is the density of the heavy and
light mass, $\theta$ is the angle between the applied
force $F$ and the distance $r$ in Eq. (5), and $a$, $b$, $c$, $d$
scaling constants given in Nace’s paper that take
values in the range $0 < a < 1$, $0 < b < 1$, $0 < c < 1$,
where $0 < d < 1$. Quoting Nace (1983) Eq. (6) is an
equation that: “disregards Stoke’s law
which gives
the rate of fall of a small sphere in a viscous fluid
under the force of gravity. It omits viscosity
and also surface tension which considers the
tension in the surface of separation between the
medium, and $\theta$ is the angle between the applied
force and the distance $r$ in Eq. (5), and $a$, $b$, $c$, $d$
scaling constants given in Nace’s paper that take
values in the range $0 < a < 1$, $0 < b < 1$, $0 < c < 1$,
and $0 < d < 1$. Quoting Nace (1983) Eq. (6) is an

\[
L_{cell} = \frac{4\pi}{3} a \ell_0 c^3 r_{BH}^3 \rho_m \sin \theta \left[ \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right] \theta_{tot} \tag{6}
\]

where $a \ell = a \ell_0$ and $\ell$ is the distance between the
theoretically and numerically, we have that the net torque exerted on the cell is

\[
\sum_{j=1}^{3} \left[ \frac{G M_E (1 + e \cos f)^2}{a_j^2 \left(1 - e^2\right)^{j/2}} \left(3 \sin^2 \phi_E - 1\right) \right] = \frac{3GM_E J_2}{2R_E^2} \left(3 \sin^2 \phi_E - 1\right) - R_E \alpha_E^2 \cos^2 \phi_E . \tag{8}
\]

furthermore, we approximate the Earth as an ellipsoid of revolution so we can write that the radius of the Earth ellipsoid becomes (Kaula, 2000):

\[
R_E = R_E \left\{ 1 - \left( f' + \frac{3}{2} f'' \right) \sin^2 \phi_E + \frac{3}{2} f'' \sin^2 \phi_E - ... \right\} = R_E \left( 1 - f' \sin^2 \phi_E \right) \tag{9}
\]

where $R_E$ is the equatorial radius of the Earth ellipsoid and where $f'$ is the Earth’s flattening given by ,

\[
f' = \frac{R_p - R_{pol}}{R_p} = \frac{3 J_2}{2R_p^2} \alpha_E^2 = \frac{3J_2 \alpha_E^2}{2GM_E} \tag{10}
\]

(Stacey, 1977):

\[
f' = \frac{R_p - R_{pol}}{R_p} = \frac{3 J_2}{2R_p^2} + \frac{R_E \alpha_E^2}{2GM_E} \tag{11}
\]

Therefore, Eq. (7) is Nace’s equation transformed as a function of the orbital elements. Similarly, using Eq. (2) for the acceleration of gravity on the surface of the Earth becomes:

\[
\text{Mass of the Earth}\left( \frac{1}{R_E^2} \right) = \frac{3GM_E J_2}{2R_E^2} \left(3 \sin^2 \phi_E - 1\right) - R_E \alpha_E^2 \cos^2 \phi_E . \tag{8}
\]
where $R_{pol}$ is the Earth’s polar radius. Therefore with the help of Eq. (9) Eq. (7) becomes:

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{R_{eq}^2 \left[ 1 - f' \sin^2 \phi_E \right]^3} \left[ \frac{3GM_E J_2}{2R_{eq}^2 \left( 1 - f' \right)^2} - \frac{3GM_E J_2}{2R_{eq}^2 \left( 1 - f' \right)^2} \right] \left( 3 \sin^2 \phi_E - 1 \right).$$  \hspace{1cm} (10)

Using Eq. (10) on the surface of the Earth and for the geocentric latitudes $\phi_E = 0^\circ, 45^\circ,$ and $90^\circ$, we obtain the following three corresponding torque expressions:

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{R_{eq}^2 \left[ 1 - f' \sin^2 \phi_E \right]^3} \left( 3 \sin^2 \phi_E - 1 \right),$$  \hspace{1cm} (11)

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{R_{eq}^2 \left[ 1 - f' \sin^2 \phi_E \right]^3} \left( 3 \sin^2 \phi_E - 1 \right),$$  \hspace{1cm} (12)

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{R_{eq}^2 \left[ 1 - f' \sin^2 \phi_E \right]^3} \left( 3 \sin^2 \phi_E - 1 \right).$$  \hspace{1cm} (13)

**TORQUE IN CELLS IN VARIOUS SPACECRAFT ORBITS**

We first consider circular orbits, i.e., eccentricity $e = 0$ with inclinations $i = 0^\circ, 45^\circ,$ and $90^\circ$, and semimajor axis $a_s$. For circular orbits the true anomaly is undefined because the orbits do not possess a uniquely determined periapsis (perigee point), and therefore the argument of latitude $u$ is used instead. In particular, circular equatorial orbits $i = 0^\circ$ do not have an ascending node point from which $u$ is defined. In this case, Earth’s equatorial plane is used as the reference plane, and the First Point of Aries as the origin of longitude and the *mean longitude* is used instead. This is the longitude that an orbiting body would have if its orbit were circular and its inclination $i = 0$. *Mean longitude* is given by the following relation $L = M + \omega + \Omega$, where $M$ is the mean anomaly defined as the angle between the perigee and the satellite radius vector assuming that the satellite moves with a constant angular velocity. Similarly, $\Omega$ is the right ascension of the node. On Earth it is measured positively (counter clockwise) in the equatorial plane from the longitude zero meridian ($\lambda = 0$) and the point of the orbit at which the satellite crosses the equator from south to north (ascending node). In this case Eq. (7) must have *mean longitude* used rather that argument of latitude. Using Eq. (7) for $L = 0^\circ$ we obtain Eq. (14), and we find the following expressions for the torque at orbital inclinations of $0^\circ, 45^\circ,$ and $90^\circ$:

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{\frac{2a_s^4}{a_s^2} \left[ 3 \sin^2 u - 1 \right],}$$  \hspace{1cm} (14)

$$L_{cell} = \frac{4\pi \ell_0 c^3 \rho_H \rho_m \sin \theta}{3} \left( \frac{b^3 \rho_H - d^3 \rho_L}{b^3 \rho_H + c^3 \rho_L d^3} \right) \frac{GM_E}{\frac{2a_s^4}{a_s^2} \left[ 3 \sin^2 u - 1 \right],}$$  \hspace{1cm} (15)
From Eq. (14) we see that in circular equatorial orbits the calculated cell torque depends on only one orbital parameter, i.e., the orbital semimajor axis $a_e$, and the geophysical parameters such as the mass of the Earth $M_E$, its radius $R_E$, and finally the $J_2$ harmonic coefficient of the gravitational field. Orbits of inclinations $i = 45^\circ$, $90^\circ$ will depend additionally on the argument of latitude $u$. Similarly, for elliptical orbits we use Eq. (7) varying only the inclination $i = 0^\circ$, $45^\circ$, and $90^\circ$ so that we obtain the following corresponding torques:

From Eq. (17)-(19) we see that in elliptical equatorial orbits the calculated cell torque depends on the following orbital parameters, namely, the orbital semimajor axis $a_e$, eccentricity $e$, the true anomaly $f$ of the orbit, and the same geophysical parameters as the circular orbits above.

**THE RELATION OF $J_2$ HARMONIC TO CELL TORQUE EXPERIMENTS**

Today's methods for the modelling and calculation of various geophysical parameters involve the continuous acceleration component monitoring of orbiting satellites. In this section we propose the use of biological and biophysical experimental parameters for the calculation, at least in principle, of parameters such as the $J_2$. As a first step, we want to establish analytical mathematical expressions that relate torque cell experimental parameters to the $J_2$ harmonic coefficient. For that we use Eq. (7) that is a modified Nase equation that calculates the torque exerted on a floating object in an experiment that takes place aboard an orbiting spacecraft. Solving Eq. (7) for $J_2$ and simplifying we obtain:

$$J_2 = \frac{2a_e^2(1-e^2)}{3R_E^2(1+e\cos f)^2}\left[1 - \frac{3a_e^2(1-e^2)^2(b^3\rho_H + c^3d^3\rho_L)}{4GM\pi c^3\alpha c_0'r_H\rho_m(1+e\cos f)^2(b^3\rho_H - d^3\rho_L)}L_{cell}\csc \theta \right].$$  (20)

This is a general equation relating the parameters of a cell torque experiment taking place aboard an orbiting spacecraft to the $J_2$ harmonic coefficient. Equation (20) can be also used in the case of circular orbits when $e = 0$. Similarly, for an experiment that takes place on the surface of Earth, solving Eq. (10) we obtain the following expression:

$$L_{cell} = \frac{4\pi \alpha \epsilon c^3 r_{BH} \rho_m \sin \theta}{3}\left(\frac{b^3\rho_H - d^3\rho_L}{b^3\rho_H + c^3\rho_L d^3}\right)\left(\frac{GM_E(1+e\cos f)^2}{a_e^2(1-e^2)^2} - \frac{3GM_E R_E^2 J_2 (1+e\cos f)^4}{2a_e^4(1-e^2)^4}\left(3\sin^2 f - 1\right)\right).$$  (17)

$$L_{cell} = \frac{4\pi \alpha \epsilon c^3 r_{BH} \rho_m \sin \theta}{3}\left(\frac{b^3\rho_H - d^3\rho_L}{b^3\rho_H + c^3\rho_L d^3}\right)\left(\frac{GM_E(1+e\cos f)^2}{a_e^2(1-e^2)^2} - \frac{3GM_E R_E^2 J_2 (1+e\cos f)^4}{2a_e^4(1-e^2)^4}\left(3\sin^2 f - 1\right)\right).$$  (18)

$$L_{cell} = \frac{4\pi \alpha \epsilon c^3 r_{BH} \rho_m \sin \theta}{3}\left(\frac{b^3\rho_H - d^3\rho_L}{b^3\rho_H + c^3\rho_L d^3}\right)\left(\frac{GM_E(1+e\cos f)^2}{a_e^2(1-e^2)^2} - \frac{3GM_E R_E^2 J_2 (1+e\cos f)^4}{2a_e^4(1-e^2)^4}\left(3\sin^2 f - 1\right)\right).$$  (19)
Equation (21) can be used at any geocentric latitude, and both equations (20)-(21). Application of Eq. (21) presupposes the ability to measure the torque applied on the floating object \( L_{cell} \), something that might be possible in future Earth and space experiments.

**CELL TORQUE TIME RATE OF CHANGE IN A SPACECRAFT EXPERIMENT**

The above derived torques are obviously changing as a function of orbital position. In order to obtain the time rate of change of the torque acting on a cell during a spacecraft orbiting experiment, let us assume that the distance \( \ell_0 \) and the angle \( \theta \) within the cells set up as given by Nace (1983) remain constant for a spacecraft revolution around the planet (Earth in our case). Let us also assume that the rest of the orbital elements in Eq. (4) do not significantly change in one spacecraft revolution, and consider that the acceleration of gravity \( g \) is a function of time \( t \)

\[
f = M + 2e \sin M + \frac{5e^2}{4} \sin 2M = nt + 2e \sin(nt) + \frac{5e^2}{4} \sin(2nt). \approx nt + 2e \sin(nt),
\]

Eventually the relation of the mean anomaly \( M \) to time \( t \) is through the equation \( M = n(t - t_p) \)

(Murray and Dermott, 1999), defined as the angle between the perigee and the satellite radius vector, assuming that the satellite moves with a constant angular velocity. \( t_p \) is the time at which the satellite crosses its perigee, and \( n \) is its mean angular velocity that is defined according to the equation: \( n = \left( \frac{GM_E}{a^3} \right)^{1/3} \), and, it is a measure of how fast a satellite progresses around its elliptical orbit. Therefore, taking the derivative w.r.t. time \( t \) of Eq. (7) we obtain the time rate of change of the cell torque to be:

\[
\frac{dL_{cell}}{dt} = \frac{4m}{3} c^3 \rho_H Q_0 \sin\theta,
\]

where, for convenience,

\[
Q_0 = \frac{\left(b^3 \rho_H - d^3 \rho_L\right)}{\left(b^3 \rho_H + c^3 d^3 \rho_L\right)} \rho_m.
\]

Next, to proceed with the time rate of change of the torque exerted on the cell around the orbit we note that the orbit element that changes with time is the true anomaly \( f \) that is included in Eq. (4). This is considered as a function of time using the relation between the time-dependent true anomaly \( f \) and the true anomaly \( M \) (Murray and Dermott, 1999):

\[
f \approx M + 2e \sin M + \frac{5e^2}{4} \sin 2M = nt + 2e \sin(nt) + \frac{5e^2}{4} \sin(2nt). \approx nt + 2e \sin(nt),
\]

Eventually the relation of the mean anomaly \( M \) to time \( t \) is through the equation \( M = n(t - t_p) \)

(Murray and Dermott, 1999), defined as the angle between the perigee and the satellite radius vector, assuming that the satellite moves with a constant angular velocity. \( t_p \) is the time at which the satellite crosses its perigee, and \( n \) is its mean angular velocity that is defined according to the equation: \( n = \left( \frac{GM_E}{a^3} \right)^{1/3} \), and, it is a measure of how fast a satellite progresses around its elliptical orbit. Therefore, taking the derivative w.r.t. time \( t \) of Eq. (4) after the substitution of \( f \) as a function of time \( t \) from equation (24), the derivative of the orbital acceleration \( \dot{g}_{total} \) to \( O(e) \) becomes:

\[
\dot{g}_{total} = e + O(e^2),
\]

\[
\frac{2GM_E B \sin A - 9GM_E R_E^2 J_2 B \sin 2Asin^2 i - 6GM_E R_E^2 J_2 B \sin A}{a_i^3 \left(1 - e^2\right)^4} - \frac{2a_i^3 \left(1 - e^2\right)^3}{a_i^3 \left(1 - e^2\right)^4} - \frac{a_i^3 \left(1 - e^2\right)^4}{a_i^3 \left(1 - e^2\right)^4} \left[ \frac{2a_i^3 \left(1 - e^2\right)^4}{a_i^3 \left(1 - e^2\right)^4} + \frac{a_i^3 \left(1 - e^2\right)^4}{a_i^3 \left(1 - e^2\right)^4} \right] e + O(e^2),
\]
where:

\[ A = nt + e\sin(nt), \quad (26) \]

\[ B = n + en\cos(nt) \quad (27) \]

\[ O(e^2) \] represents negligible higher-order terms.

For circular orbits of inclinations \( i = 0^\circ, 45^\circ, \) and \( 90^\circ \) we obtain, respectively:

\[ \frac{dL_{cell}}{dt} = 0 \]

\[ \frac{dL_{cell}}{dt} = -6\pi c^3 l_{bh}^3 \ell_0 Q_0 \sin\theta \left( \frac{GM_E R_E^2 J_2 B \sin 2A}{a_i^3} \right) \quad (28) \]

\[ \frac{dL_{cell}}{dt} = -36\pi c^3 l_{bh}^3 \ell_0 Q_0 \sin\theta \left( \frac{GM_E R_E^2 J_2 B \sin 2A}{a_i^3} \right) \quad (29) \]

and therefore for elliptical orbits of the same inclinations \( i = 0^\circ, 45^\circ, \) and \( 90^\circ \) as above, we obtain:

\[ \frac{dL_{cell}}{dt} = \frac{4\pi l}{3} \ell_0 c^3 l_{bh}^3 Q_0 \sin\theta \left[ \frac{2GM_E B \sin A}{a_i^2 \left(1-e^2\right)} - \frac{6GM_E R_E^2 J_2 B \sin 2A}{a_i^4 \left(1-e^2\right)^3} \right] e + O(e^2), \quad (30) \]

\[ \frac{dL_{cell}}{dt} = \frac{4\pi l}{3} \ell_0 c^3 l_{bh}^3 Q_0 \sin\theta \left[ -\frac{2GM_E B \sin A}{a_i^2 \left(1-e^2\right)^2} + \frac{9GM_E R_E^2 J_2 B \sin 2A}{4a_i^4 \left(1-e^2\right)^4} - \frac{6GM_E R_E^2 J_2 B \sin A}{a_i^4 \left(1-e^2\right)^3} \right] e + O(e^2), \quad (31) \]

\[ \frac{dL_{cell}}{dt} = \frac{4\pi l}{3} \ell_0 c^3 l_{bh}^3 Q_0 \sin\theta \left[ -\frac{2GM_E B \sin A}{a_i^2 \left(1-e^2\right)^2} + \frac{9GM_E R_E^2 J_2 B \sin 2A}{2a_i^4 \left(1-e^2\right)^4} - \frac{6GM_E R_E^2 J_2 B \sin A}{a_i^4 \left(1-e^2\right)^3} \right] e + O(e^2), \quad (32) \]

Equations (28) to (32) demonstrate that the time rate of change of the torque exerted on the cell around the orbit of a spacecraft is a periodic function of orbital time \( t. \)

**DISCUSSION AND NUMERICAL RESULTS**

For the numerical evaluation of our formulas regarding cell torques in experiments that take place on the surface of the Earth corrected for the \( J_2 \) harmonic coefficient, the rotation of the Earth, at its angular velocity \( \omega_E \) following Nace (1983) we consider the following biological objects: First, a sarcoma cell with length \( \ell_0 = 6.4 \) \( \mu m \) in peritoneal fluid for which \( \rho_m = 1.012 \) \( g/cm^3 \), with glycogen granules \( \rho_H = 1.510 \) \( g/cm^3 \) as \( m_H \) and oil vacuoles \( \rho_L = 0.918 \) \( g/cm^3 \) as \( m_L \). Similarly, the masses \( m_H \) and \( m_L \) are separated by 3/4 length of the cell \( \ell_0 \) and \( a = 3/4. \) The two masses are of equal volumes and fill the buoyant volume \( b = c = d = 1, \) are spherical with radii \( 1/8 \) of the length of
the cell \( r_{BH} = \ell_0 / 8 \) (Nace, 1983), and we take \( \theta = 90^\circ \) at the instant of observation. Furthermore, we consider a human egg of diameter 89 \( \mu \)m, for which \( \rho_m = 1.007 \) g/cm\(^3\), \( \rho_H = 1.100 \) g/cm\(^3\) as \( m_H \) and \( \rho_L = 0.950 \) g/cm\(^3\) (Nace, 1983), assuming \( a = 0.75, b = c = d = 1 \) (Nace, 1983), and finally we consider the oocyte, yolk of the chicken, \( Gallus gallus \) egg, which has a polar diameter \( \ell_0 = 31 \) mm with the blastodisc and nucleus of Pander occupying a volume with a radius \( r_L = 3 \) mm and a \( \rho_L = 1.027 \) g/cm\(^3\), respectively. The remainder of the yolk has a radius \( r_L = 12.5 \) mm and \( \rho_H = 1.032 \) g/cm\(^3\). This yolk floats in an albumin-containing medium with \( \rho_m = 1.040 \) g/cm\(^3\); furthermore, the constants \( a, b, c, d \) are 0.5, 1.0, 0.24, 1.0 (Nace, 1983), respectively. For the mass/radius of the Earth we use \( M_E = 5.94 \times 10^{24} \) kg, and \( R_E = 6378.1363 \) km, and the angular velocity of rotation is \( \omega_E = 7.292115 \times 10^{-5} \) rad/s (Vallado and McClain, 2007). The oblateness coefficient is \( J_2 = -0.0010827 \) (Kaula, 2000), and the period of an orbital revolution of the spacecraft at 300 km is \( T_{rev} = 5463.282 \) s, and its mean motion \( n = 0.001149492 \) rad/s. Our results for the calculated torque on the surface of the Earth and in orbit around the Earth are given in tables 1, 2, 3, and 4.

Next, considering the Earth as an ellipsoid we derive formulas for the calculation of the torque exerted on a human egg on the surface of the Earth as a function of the geocentric latitude \( \phi_E \). For that, using the parameters above, we obtain:

\[
L_{net,egg} = \sin \theta \left\{ -3.941 \times 10^{-11} + 3.954 \times 10^{-11} \sin^2 \phi_E - 1.321 \times 10^{-13} \sin^4 \phi_E \right. \\
\left. + \frac{1.124 \times 10^{-8}}{(1 - 0.00352 \sin^2 \phi_E)^2} - \frac{1.825 \times 10^{-11}}{(1 - 0.00352 \sin^2 \phi_E)^4} \right. \\
\left. + \frac{5.476 \times 10^{-11} \sin^2 \phi_E}{(1 - 0.00352 \sin^2 \phi_E)^6} \right\}, \tag{33}
\]

Similarly, extending this on the Martian surface ellipsoid we similarly derive:

\[
L_{net,egg} = \sin \theta \left\{ -1.982 \times 10^{-11} + 1.993 \times 10^{-11} \sin^2 \phi_M - 1.167 \times 10^{-13} \sin^4 \phi_M \right. \\
\left. + \frac{1.270 \times 10^{-11}}{(1 - 0.00588 \sin \phi_M)^2} - \frac{4.310 \times 10^{-9}}{(1 - 0.00588 \sin^2 \phi_M)^4} \right. \\
\left. + \frac{3.810 \times 10^{-11} \sin^2 \phi_M}{(1 - 0.00588 \sin^2 \phi_M)^6} \right\} \sin \theta, \tag{34}
\]

where \( \phi_M \) is the areocentric latitude, defined north and south of the Martian equator, from 0° to 90° to 0° to -90°. In Figure 3 below we plot the effect of the geocentric latitude \( \phi_E \) effect on the torque exerted on a human egg, for various values of the angle \( \theta = 0^\circ, 88^\circ, 86^\circ, 84^\circ, 82^\circ, 80^\circ \). Table 1 tabulates the results for the net torque for an experiment that takes place on the surface of the Earth.
Table 1. Torque geocentric latitude effect exerted on various test objects.

<table>
<thead>
<tr>
<th>Geocentric Latitude $\varphi$ [$^\circ$]</th>
<th>Cell length $\ell_0$ [$\mu$m]</th>
<th>Torque $L$ [dyne cm]</th>
<th>Equivalent to torque energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Sarcoma cell</td>
<td>$9.107 \times 10^{-10}$</td>
<td>0.09107 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.126 \times 10^{-10}$</td>
<td>0.09126 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.145 \times 10^{-10}$</td>
<td>0.09145 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.164 \times 10^{-10}$</td>
<td>0.09164 $\mu$J</td>
</tr>
<tr>
<td></td>
<td>Human Egg</td>
<td>Nace’s result $1.5 \times 10^{-8}$ dyne cm</td>
<td>$1.118 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.120 \times 10^{-8}$</td>
<td>1.120 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.123 \times 10^{-8}$</td>
<td>1.123 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.125 \times 10^{-8}$</td>
<td>1.125 $\mu$J</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.128 \times 10^{-8}$</td>
<td>1.128 $\mu$J</td>
</tr>
<tr>
<td></td>
<td>Gallus gallus egg</td>
<td>Nace’s result 0.85 dyne cm</td>
<td>0.818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.820</td>
<td>82.031 nJ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.822</td>
<td>82.204 nJ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.824</td>
<td>82.380 nJ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.825</td>
<td>82.550 nJ</td>
</tr>
</tbody>
</table>

Figure 3. Torque exerted on a human egg in an experiment taking place on the surface of the Earth as a function of geocentric latitude $\varphi_E$ and for various angles $\theta = 90^\circ$ (blue), $88^\circ$ (red), $86^\circ$ (yellow brown), $84^\circ$ (light blue), $82^\circ$ (purple), $80^\circ$ (light green) between the force and distance $d$. 
Figure 4. Torque exerted on a human egg in an experiment taking place on the surface of Mars as a function of areocentric latitude $\phi_E$ and for various angle $\theta = 90^\circ$ (blue), $88^\circ$ (red), $86^\circ$ (yellow brown), $84^\circ$ (light blue), $82^\circ$ (purple), $80^\circ$ (light green) between the force and distance $d$.

Figure 5. Plot of the torque exerted on cells of increasing length having the same properties as those given by Nace (1983) on the surface of the Earth as a function of geocentric latitude $\phi_E$ and the length of the cell $\ell_0$, assuming $\theta = 90^\circ$. 
Higher θ angles result in higher torques. Similarly, Figure 4 demonstrates torques exerted on a human egg in an experiment taking place on the surface of Mars as a function of areocentric latitude φE resulting in behavior similar to but less by approximately an order of magnitude when compared to the torque applied in an Earth experiment. Higher torques are expected if the experiment takes place in a relatively low orbit scenario (i.e., around Earth or Mars). In very deep space and far away from the Earth g does not become identically zero and therefore there will always be torque acting on the cells. Finally, the imparted torque is highly sensitive to the size of the cell to the fourth power, but also to the distribution of its subcellular particulates. Figure 5 demonstrates the torque exerted on cells of increasing size in the scenario having the same properties as those cells given by Nace (1983) on the surface of the Earth, as a function geocentric latitude φE and cell’s length ℓ₀, assuming θ = 90°. We see that the torque increases simultaneously as the cell’s length ℓ₀ and geocentric (planetocentric) latitude φE increase.

In Nace’s paper the author obtains results for a sarcoma cell experiment on the surface of the Earth in which g is taken as 980.0 cm/s², a near-average value that was considered suitable for the purpose. We obtain results using our modified Nace expression for the torque, corrected for the J₂ harmonic and also the rotation of the Earth. We find that this introduces a smaller torque with a % difference in the range 2.24% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 1.40% if the corresponding geocentric latitude range is in the range 0° ≤ φE ≤ 90°. At this point we remind the reader about the peculiarity of our first inequality, sequenced to match the second, since lower latitudes result in higher percentage differences and higher latitudes result in lower percentage differences when comparing to Nace’s result. Our pole calculated value results in a 1.40% difference less than the result given by Nace for the same cell that has length equal of 6.4 μm. At the same time our calculations demonstrate that higher torque is exerted on the cell at the poles due to the higher value of g. In the case of a human egg and for the same geocentric latitude range, we find a % difference in the range of 29% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 28%. This results from the fact that in our calculations we have used \( r_BH = \ell_0 / 5 \), where Nace seems to have used \( r_BH = \ell_0 / 4.54 \). It appears that the torque results in Nace’s original formula and also in the one derived in this paper greatly depend on the choice of parameter \( r_BH \), on the surface of the Earth and also in space. Our polar torque experiment value exhibits a 28% difference less than the one given by Nace, but still consistent with a higher torque value, for higher polar g scenario. Finally, for the same geocentric latitude range the Gallus gallus egg cell results in percentage difference in the range 3.8% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 2.9%. Next, and for the same type of cells aboard an orbiting spacecraft in circular (e = 0) and elliptical orbits (e = 0.2) and inclinations \( i = 0°, 45° \), and 90°, we obtain the following % differences between surface and orbital values: Nace’s “sarcoma” cell 11% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 10%, and also for a 50 μm sarcoma cell, we also obtain 8.8% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 9.2%, assuming that all the parameters remain the same. Similarly, for Nace’s “sarcoma” cell in an elliptical orbit, we obtain 2.9% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 2.4%, and also for the 50 μm sarcoma cell 0.6% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 1.0%, respectively. Next, for the human egg in the same orbital scenarios we obtain 38% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 37%, and 30% ≤ [ΔL(L)ₜ₉₉]⁻¹ ≤ 29%, respectively. Finally, for the Gallus gallus egg we obtain...
12.5% \leq \left| \frac{AL}{L} \right|_{\text{egg}}^{r=0} \leq 12.1\%,  
6\% \leq \left| \frac{AL}{L} \right|_{\text{egg}}^{r=0.2} \leq 5.6\%, \text{ respectively.} \]

We find that elliptical orbits and highly elliptical orbits result to a less % difference, when compared with Nace’s results. This is due to the fact that elliptical orbits exhibit higher values of orbital acceleration $g$. It is possible by adjusting the eccentricity of the orbit, to achieve a torque effect similar to the torque exerted on the cell under a constant gravitational acceleration on the surface of the Earth as given by Nace (1983). For example, an elliptical polar orbit $i = 90^\circ$ at the orbital altitude of 300 km would need to achieve eccentricities $e = 0.211$ (elliptical), and $e = 1.398$ (hyperbolic) in order that the torque felt by a *Gallus gallus* egg in the orbital experiment around the Earth at the given eccentricities would result in a torque exerted on the cell that is equal to that exerted at the surface of the Earth. Figure 5 demonstrates the torque exerted on a human egg in the scenario given by Nace (1983) for an experiment that takes place in elliptical polar orbit ($e = 0.01$) at the orbital altitude $h = 300$ km, as a function of semimajor axis $a$, and the orbital true anomaly $f$, assuming $\theta = 90^\circ$. Similarly, Figure 7 demonstrates of the torque exerted on a human egg in the scenario given by Nace (1983) for an experiment that is taking place in a polar orbiting spacecraft at $h = 300$ km and eccentricity $e = 0.1$ as a function of the angle $\theta$ and the orbital true anomaly $f$. Similarly, figures 8 and 9 plot the torque exerted on a human egg in an experimental scenario taking place aboard a polar orbiting spacecraft at $h = 300$ km and eccentricity $e = 0.4$, as a function of the angle $\theta$ and the orbital true anomaly $f$. Finally, Figure 10 demonstrates the variation of $g$ at $h = 300$ km along the orbit of the spacecraft for the time of one orbital period. The exerted torque exhibits a periodic effect in the true anomaly, with a minimum around $f = 180^\circ$ and reduces as the semimajor axis of the spacecraft increases. Finally, for the *Gallus gallus* egg we find that $0.818$ dynes cm $\leq L_{\text{Gal}} \leq 0.825$ dynes cm. On the other hand, as another example we quote Berg (2003) and comparing we say that the torque that a human egg feels on the surface of the Earth at geocentric latitude $\phi_E$ = 45$^\circ$ in Nace’s scenario, when compared to the torque produced by a flagellar motor of an *E. coli* bacterium, is 245 to 414 times smaller or $L_{\text{egg}}=(245-414)L_{E,Coli}$. Similarly, for a sarcoma cell we have that $L_{\text{sar}} \approx (20-34)L_{E,Coli}$, where both torques have been expressed in pN nm. Finally, for a *Gallus gallus* egg we obtain that $L_{\text{Gal}} = (0.303-1.80) \times 10^{10}$ $L_{E,Coli}$, respectively. Equations (35), (36), (37) give the torques exerted on a human egg, a *Gallus gallus* egg, and a sarcoma cell on the surface of a planetary body in the solar system, as a function of the body’s planetary mass $M_p$, oblateness coefficient $J_2$, planetocentric latitude $\phi_p$, and also its planetary flattening coefficient , and finally the angle $\theta$ to be:

\[
L_{\text{egg}} = \sin \theta \left[ \frac{7.750 \times 10^{-19} M_p}{R_{eq}^2 \left( 1 - f_p \sin^2 \phi_p \right)} \right] - 1.162 \times 10^{-18} \frac{M_p J_2 \left( 3 \sin^2 \phi_p - 1 \right)}{R_{eq}^2 \left( 1 - f_p \sin \phi_p \right)^4} - 1.162 \times 10^{-11} R_{eq}^2 \omega_p^2 \cos^2 \phi_p \left( 1 - f_p \sin^2 \phi_p \right), \quad (35)
\]

\[
L_{\text{Gal--gal}} = \sin \theta \left[ \frac{5.882 \times 10^{-11} M_p}{R_{eq}^2 \left( 1 - f_p \sin^2 \phi_p \right)} \right] - 8.823 \times 10^{-11} M_p J_2 \left( 3 \sin^2 \phi_p - 1 \right) - 8.820 \times 10^{-4} \frac{R_{eq}^2 \omega_p^2 \cos^2 \phi_p \left( 1 - f_p \sin^2 \phi_p \right)}{R_{eq}^2 \left( 1 - f_p \sin \phi_p \right)^4}, \quad (36)
\]
\[ L_{sar} = \sin \theta \left[ \frac{6.312 \times 10^{-20} M_p}{R_{eq}^2 \left( 1 - f_p \sin \phi_p \right)} - \frac{9.467 \times 10^{-20} M_p f_p^3 \left( 3 \sin^2 \phi_p - 1 \right)}{R_{eq}^2 \left( 1 - f_p \sin \phi_p \right)^3} - 9.463 \times 10^{-13} R_{eq}^2 \omega_p^2 \cos^2 \phi_p \left( 1 - f_p \sin^2 \phi_p \right) \right]. \] (37)

Next, we consider the same experiment as above in a spacecraft in circular/elliptical orbits around Earth with inclinations \( i = 0^\circ, 45^\circ, 90^\circ \), at the altitude \( h = 300 \) km. Results are tabulated in tables 2-5:

**Table 2. Torque effect exerted on various test objects in an experiment taking place in a spacecraft in circular orbit around Earth.**

<table>
<thead>
<tr>
<th>Orbital Eccentricity</th>
<th>Cell length ( \ell_0 ) [( \mu )m]</th>
<th>Torque ( L ) [dyne cm]</th>
<th>Equivalent Energy to torque</th>
</tr>
</thead>
<tbody>
<tr>
<td>( e = 0 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>Sarcoma cell</td>
<td>8.340 \times 10^{-10}</td>
<td>0.0834 fJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td>50</td>
<td>8.356 \times 10^{-10}</td>
<td>0.0835 fJ</td>
</tr>
<tr>
<td>( i = 90^\circ )</td>
<td>8.375 \times 10^{-10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( e = 0 )</td>
<td>Human Egg</td>
<td>1.024 \times 10^{-8}</td>
<td>1.024 fJ</td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>89</td>
<td>1.026 \times 10^{-8}</td>
<td>1.026 fJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td>1.028 \times 10^{-8}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( e = 0 )</td>
<td>Gallus gallus egg</td>
<td>0.750</td>
<td>75.000 nJ</td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>31000</td>
<td>0.753</td>
<td>75.300 nJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( i = 90^\circ )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Torque effect exerted on various test objects in an experiment taking place in a spacecraft in a slightly elliptical orbit (\( e = 0.01 \)) around Earth.**

<table>
<thead>
<tr>
<th>Orbital Eccentricity</th>
<th>Cell length ( \ell_0 ) [( \mu )m]</th>
<th>Torque ( L ) [dyne cm]</th>
<th>Equivalent Energy to torque</th>
</tr>
</thead>
<tbody>
<tr>
<td>( e = 0.01 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>Sarcoma cell</td>
<td>8.340 \times 10^{-10}</td>
<td>0.0834 fJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td>50</td>
<td>8.360 \times 10^{-10}</td>
<td>0.0836 fJ</td>
</tr>
<tr>
<td>( i = 90^\circ )</td>
<td>8.376 \times 10^{-10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( e = 0 )</td>
<td>Human Egg</td>
<td>1.024 \times 10^{-8}</td>
<td>1.024 fJ</td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>89</td>
<td>1.026 \times 10^{-8}</td>
<td>1.026 fJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td>1.028 \times 10^{-8}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( e = 0.01 )</td>
<td>Gallus gallus egg</td>
<td>0.750</td>
<td>75.000 nJ</td>
</tr>
<tr>
<td>( i = 0^\circ )</td>
<td>31000</td>
<td>0.751</td>
<td>75.100 fJ</td>
</tr>
<tr>
<td>( i = 45^\circ )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( i = 90^\circ )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Torque effect exerted on various test objects in an experiment taking place in a spacecraft in an elliptical orbit ($e = 0.2$) around Earth.

<table>
<thead>
<tr>
<th>Orbital Eccentricity $e = 0.2$</th>
<th>Cell length $\ell_0$ [μm]</th>
<th>Torque $L$ [dyne cm]</th>
<th>Equivalent Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>$i = 0^\circ$ Sarcoma cell</td>
<td>50</td>
<td>$9.045 \times 10^{-10}$</td>
<td>0.0904 fJ</td>
</tr>
<tr>
<td>$i = 45^\circ$</td>
<td>50</td>
<td>$9.067 \times 10^{-10}$</td>
<td>0.0906 fJ</td>
</tr>
<tr>
<td>$i = 90^\circ$ Human Egg</td>
<td>89</td>
<td>$9.089 \times 10^{-10}$</td>
<td>0.0908 fJ</td>
</tr>
<tr>
<td>$e = 0.2$</td>
<td>Human Egg</td>
<td>$1.110 \times 10^{-8}$</td>
<td>1.110 fJ</td>
</tr>
<tr>
<td>$i = 0^\circ$ Gallus gallus egg</td>
<td>31000</td>
<td>$0.800$</td>
<td>80.00 nJ</td>
</tr>
<tr>
<td>$i = 45^\circ$</td>
<td>$0.803$</td>
<td>80.30 nJ</td>
<td></td>
</tr>
<tr>
<td>$i = 90^\circ$</td>
<td>$0.804$</td>
<td>80.40 nJ</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Torque effect exerted on various test objects in an experiment taking place in a spacecraft in an elliptical orbit ($e = 0.4$) around Earth.

<table>
<thead>
<tr>
<th>Orbital Eccentricity $e = 0.4$</th>
<th>Cell length $\ell_0$ [μm]</th>
<th>Torque $L$ [dyne cm]</th>
<th>Equivalent Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>$i = 0^\circ$ Sarcoma cell</td>
<td>50</td>
<td>$1.1809 \times 10^{-9}$</td>
<td>0.1180 fJ</td>
</tr>
<tr>
<td>$i = 45^\circ$</td>
<td>50</td>
<td>$1.1846 \times 10^{-9}$</td>
<td>0.1184 fJ</td>
</tr>
<tr>
<td>$i = 90^\circ$ Human Egg</td>
<td>89</td>
<td>$1.1883 \times 10^{-9}$</td>
<td>0.1188 fJ</td>
</tr>
<tr>
<td>$e = 0.2$</td>
<td>Human Egg</td>
<td>$1.4499 \times 10^{-8}$</td>
<td>1.4499 fJ</td>
</tr>
<tr>
<td>$i = 0^\circ$ Gallus gallus egg</td>
<td>31000</td>
<td>$1.0615$</td>
<td>106.150 nJ</td>
</tr>
<tr>
<td>$i = 45^\circ$</td>
<td>$1.0648$</td>
<td>106.480 nJ</td>
<td></td>
</tr>
<tr>
<td>$i = 90^\circ$</td>
<td>$1.0681$</td>
<td>106.810 nJ</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6. Plot of the torque exerted on a human egg in the scenario given by Nace (1983), for an experiment that is taking place aboard a spacecraft in an elliptical polar orbit around the Earth, with $e = 0.01$ and at the orbital altitude $h = 300$ km, as a function of orbital semimajor axis $a$ and the orbital true anomaly $f$, assuming $\theta = 90^\circ$. 

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Haranas et al. -- Mechanics of Gravity-induced Torque on Cells
Figure 7. Plot of the torque exerted on a human egg in the scenario given by Nace (1983) for an experiment that is taking place in a polar orbiting spacecraft at $h = 300 \text{ km}$ and eccentricity $e = 0.1$ as a function of the angle $\theta$ and the orbital true anomaly $f$.

Figure 8. Plot of the torque exerted on a human egg in the scenario given by Nace (1983) for an experiment that is taking place in a polar orbiting spacecraft at $h = 300 \text{ km}$ and eccentricity $e = 0.4$ as a function of the angle $\theta$ and the orbital true anomaly $f$. 
Figure 9. Plot of the torque exerted on a human egg in the scenario given by Nace (1983) for an experiment that is taking place in a spacecraft in polar circular orbit at $h = 300$ km as a function of the angle $\theta$ and the orbital true anomaly $f$.

Figure 10. Plot of the variation of gravitational orbital acceleration as a function of orbital time $t$ of a full orbit at the orbital altitude of 300 km and for various eccentricities.
CONCLUSIONS

In this paper we have studied the effect of orbit parameters on the torque exerted by gravity on cells and eggs. In particular we have calculated the torque effects, in the human egg, sarcoma, and *Gallus gallus* egg. We have extended Nace’s model on the surface of the Earth by considering the *J₂* harmonic, the rotation of the Earth, and its flattening. This is for an experiment that takes place on the surface of the Earth at various geocentric latitudes. The torque acting in cells is proportionally related to the acceleration of gravity *g*, and the fourth power of the cell’s length. Furthermore, we have also extended Nace’s result for an experiment that takes place in orbit around a planetary body. We have found that in orbit around Earth, the effect of torque is less when compared to that of the surface, because gravity is less but not zero at the orbital altitude of the spacecraft, and that the effect of the *J₂* harmonic can be calculated for possible future experiments. We have derived expressions for the applied cell torques by Earth and Mars gravity, taking their flattening into account. We have also found that elliptical polar orbits result in higher torques, and that high eccentricities increase the torque drastically. Depending on the size of the cell, percentage differences due to the correction of *J₂* harmonic and rotation of the Earth that are less than 1% can be ignored, where higher effects should be included.

ACKNOWLEDGEMENTS

The authors want to thank the Editor-in-Chief of *Gravitational and Space Biology*, Dr. Anna-Lisa Paul, who with her valuable comments, suggestions, and encouraging correspondence helped in the improvement of the paper’s final version.

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The authors want to thank Ivana Haranas for redesigning Nace’s original diagram so that it could be used in this paper.

NOMENCLATURE (in order of appearance in the text)

- *aₜ*: orbital semimajor axis.
- *e*: eccentricity of the orbit.
- *u*: argument of latitude.
- *f*: true anomaly.
- *G*: constant of universal gravitation.
- *i*: orbital inclination.
- *r’*: radial orbital distance of the spacecraft from the center of the Earth.
- *J₂*: oblateness coefficient of the Earth.
- *M*: the mass of the Earth.
- *Mₚ*: mass of any planet.
- *Vₑ*: total gravitational potential of the Earth.
- *x₁, y₁, z₁*: define a right handed coordinate system.
- *F*: applied force.
- *r*: distance of the center of mass that the force is applied to the axis of rotation.
- *ℓ*: distance between the centers of the heavy and light masses in the cell.
- *ℓ₀*: total cell length.
- *r_H* = *brₜBH* where, where *r_H* is the radius of the heavy mass.
- *rₜBH*: the radius of the cell.
- *ρₚ*: density of the medium.
- *ρ_H*: density of the heavy mass.
- *ρ_L*: density of light mass.
- *a, b, c, d*: constants in the range [0,1].
- *g_{tot}*: corrected gravitational acceleration.
- *L*: torque.
- *Rₑ*: radius of the Earth.
- *Rₑq*: equatorial radius of the Earth.
- *Rₚol*: polar radius of the Earth.
- *f’*: flattening of the Earth.
Haranas et al. -- Mechanics of Gravity-induced Torque on Cells

$f_M$ flattening of Mars.

$f_p$ planetary flattening.

$M$ orbital mean anomaly.

$n$ spacecraft mean angular velocity.

$\phi_E$ geocentric latitude.

$\omega_E$ angular velocity of the Earth.

$\theta_E$ colatitude.

$\omega$ argument of the perigee.

$\Omega$ argument of the ascending node.

$\lambda$ geocentric longitude.

$\theta$ is the angle between $F$ and $r$.

$L = M + \Omega + \omega$ mean longitude.

REFERENCES


The Effects of Gamma and Proton Radiation Exposure on Hematopoietic Cell Counts in the Ferret Model

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ABSTRACT

Exposure to total-body radiation induces hematological changes, which can detriment one’s immune response to wounds and infection. Here, the decreases in blood cell counts after acute radiation doses of \(\gamma\)-ray or proton radiation exposure, at the doses and dose-rates expected during a solar particle event (SPE), are reported in the ferret model system. Following the exposure to \(\gamma\)-ray or proton radiation, the ferret peripheral total white blood cell (WBC) and lymphocyte counts decreased whereas neutrophil count increased within 3 hours. At 48 hours after irradiation, the WBC, neutrophil, and lymphocyte counts decreased in a dose-dependent manner but were not significantly affected by the radiation type (\(\gamma\)-rays verses protons) or dose rate (0.5 Gy/minute verses 0.5 Gy/hour). The loss of these blood cells could accompany and contribute to the physiological symptoms of the acute radiation syndrome (ARS).

INTRODUCTION

The effects of exposure to ionizing radiation (IR) are of interest to the space exploration community as well as patients considering radiotherapy. IR has a sufficient amount of energy to induce physical symptomatology within minutes of exposure, appearing as the acute radiation syndrome (ARS). The prodromal phase of ARS includes nausea, vomiting, and fatigue. The quality of radiation, dose, and dose-rate are all contributing factors to the differential symptoms of ARS. These prodromal symptoms can be followed by a dramatic decrease in peripheral blood cell counts, as hematopoietic cells represent a renewal system consisting of cells with fast division rates that are known to be sensitive to IR.

Astronauts are exposed to chronic low-dose and low-dose rate IR during low-Earth orbit missions (Badhwar, 2002; Cucinotta et al., 2008). Contributing to low-Earth orbit radiation exposures are solar particle events (SPEs), which accelerate ions and release unpredictable doses of IR; therefore, SPE radiation exposure poses a threat to astronauts in a spacecraft where shielding...
Sanzari et al. -- Gamma and Proton Radiation Effects on Blood Cell Counts

is available, and especially during an extravehicular activity (EVA), in which shielding may only be provided by the space suit. SPE radiation consists predominantly of energetic proton particles with energies greater than 10 millielectron volts (MeV). The duration of radiation exposure from an SPE can last several days. It has been estimated that the largest dose of SPE radiation recorded from a historically large SPE (August 1972) was capable of delivering a 1.38 Gy dose (during an EVA) and a 0.46 Gy dose (in a spacecraft) to the blood forming organs to astronauts (Hu et al., 2009).

The Center of Acute Radiation Research (CARR) has been established to investigate the risks of ARS induced by SPE-like radiation, and develop countermeasures for those risks if appropriate. Thus, measuring the early effects of SPE radiation exposure in the hematopoietic system in response to space-relevant radiation is a focus. The hematological effects of space radiation contribute to the compromised immune defense in astronauts observed in the space environment as well as upon landing (Sonnenfeld et al., 2003; Sonnenfeld and Shearer, 2002; Crucian et al., 2008). The complications associated with the hematopoietic syndrome include infection and internal hemorrhage. The decrease in peripheral blood cell counts recorded within the first 48 hours of radiation exposure serve not only as a marker for the severity of the exposure, but also as a marker for treatment and prognosis.

As part of this work, we have previously evaluated the acute effects of gamma and proton radiation in the murine model (Maks et al., 2011; Ware et al., 2010). Recently, we reported increased blood clotting times in ferrets exposed to SPE-like proton radiation, which may contribute to radiation-induced coagulopathies (Krigsfield et al., 2012). In the current report, we investigated the effects of SPE-like proton radiation on the hematopoietic system in the ferret model. The effects of proton radiation on peripheral blood cell counts were compared to the effects produced by conventional gamma-ray (γ-ray) radiation. Effective doses and relative biological effectiveness values were determined to compare the effects of the differing types of radiation.

MATERIALS & METHODS

Animals

Female descented ferrets (approximately 12-16 weeks of age) were obtained from Marshall Bioresources (North Rose, NY) and given an acclimation period of 7 days at the Loma Linda University Medical Center (LLUMC). Animals were group-housed and provided access to food and water ad libitum with a 12 hour light-dark cycle. The animals were maintained under standard husbandry conditions and all procedures for the animal care and treatment were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Pennsylvania and the LLUMC. Ferrets were randomly assigned to treatment groups, with each group consisting of 6–12 animals.

Physics and Dosimetry

γ-Ray radiation was chosen as the reference radiation for the determination of relative biological effectiveness (RBE) values and was delivered using a 60Co source (Eldorado Model ‘G’ machine, Atomic Energy of Canada Ltd., Commercial Products Division, Ottawa, Canada) at the LLUMC. For radiation exposures at a high dose rate (HDR) of 0.5 Gy/minute, the source to target distance was 150 cm, with a usable radiation field of 40 x 40 cm² and a field flatness of 5.4% in the horizontal direction and 3.6% in the vertical direction. No additional material was placed between the source and target to attenuate the flux of the beam. Depth dose measurements were made using a calibrated PTW Markus ionization chamber for comparison with proton irradiation.

For the proton radiation exposures, experiments were performed in the LLUMC horizontal clinical beam-line using an incident beam of 155-MeV. The incident protons were scattered into a uniform field using the clinical 2-stage scattering system and modulated in depth using an 11-cm clinical modulator wheel. At the
exit of the beam-line, the beam was degraded to
the required energy using a pre-determined
thickness of polystyrene. The proton experiments
used two different apertures to target distances
and two different polystyrene thicknesses to
achieve the desired beam sizes and dose rates of
0.5 Gy/minute (HDR) or 0.5 Gy/hour (LDR),
respectively. For the LDR proton experiments, the
animal cages were placed 122 cm downstream of
isocenter. The useable beam at this distance was
measured as 22 x 22 cm² while achieving a
flatness of ±10%. This field size allowed only
one ferret in the radiation chamber (length, width,
and height was approximately 24 x 16 x 9 cm) to
be irradiated. For the HDR proton experiments,
the animal cages were placed at isocenter. The
usable beam at this distance was experimentally
verified and measured as 19 x 19 cm², again
allowing only one ferret in the chamber to be
irradiated. The upstream polystyrene degrader was
tuned to achieve a fully modulated proton beam of
110-MeV at the inside of the irradiation chamber.
Depth dose profiles were measured for the
optimized polystyrene degrader thickness using
Gafchromic film, type MD-55, and verified with a
PTW Markus ionization chamber.

To complete a more efficient proton radiation
of multiple ferrets at the LDR, an alternate
scattering system was developed and verified in
the LLUMC Proton Research Room. The incident
protons were scattered using a 2-stage scattering
system to a useable radiation field of 50 cm
diameter with flatness and depth dose profiles that
were comparable to the clinical system described
above. This system allowed for 12 ferrets to be
irradiated at any one time.

γ-Ray or Proton Radiation Exposure

For the γ-ray and the proton radiation
experiments, the animals were placed in
Plexiglass radiation chambers measuring
approximately 24 x 16 x 9 cm in length, width and
height, respectively. The custom-made radiation
chambers contained adequate holes for proper air
circulation and animals were provided with
NapaNectar hydrating gel (SE Lab Group Inc.,
Napa, CA). For the LDR experiments, the
chambers were slightly modified to contain a
watering system on the outside of each chamber
that did not compromise the radiation dose
delivered to each animal, so these animals were
not provided NapaNectar gel. The LDR chambers
also contained a thermistor which enabled real-
time monitoring of temperature within the
radiation chamber during the long LDR
exposures. The average recorded temperature of
an empty chamber prior to radiation was 70°C,
and the average recorded temperature during/after
the HDR and LDR radiation exposures (with an
animal in the chamber) was 75°C; thus, these
results indicated that the temperature during the
containment remained adequate. The animals
were not anesthetized during irradiation to total
doses ranging from 0.75 Gy to 2.0 Gy and were
able to express normal postural movements
throughout. For the HDR experiments, although
the actual radiation exposures were only minutes
long (as opposed to hours in the case of the LDR
experiments), the animals were restrained in the
radiation chamber for the same amount of time as
the LDR animals, corresponding to the
appropriate dose.

Complete Blood Cell Count Analyses

Animals were anesthetized by isoflurane
inhalation for blood collection. Blood samples
were collected from the external jugular vein and
immediately placed in tubes containing
ethylenediaminetetraacetic acid (EDTA). Blood
draws were performed on each animal at least 2
days prior to radiation exposure (Pre) and again at
3 hours (3 h) and 48 hours (48 h) post-irradiation.
A complete blood count (CBC) with differential
was performed using a Bayer Advia 120
Hematology Analyzer within 24 hours of blood
collection (Antech Diagnostics, Irvine, CA).

Statistical Analyses

The average counts of white blood cells
(WBC), neutrophils, lymphocytes, monocytes and
eosinophils were determined prior to the radiation
exposure as baseline values. The blood cell
counts obtained in animals at different time points
were divided by the respective baseline values and
expressed as fractions of control for statistical
analyses. The effects of experimental factors
(Radiation Type, Dose Rate, and Dose) and their
interactions on the blood cell counts were
determined by variance analyses using a general
linear model, which was performed using a
Minitab statistical software, release 15 (Minitab
Inc., State College, PA). The relationship
between the dose and blood cell count was determined by fitting the data to a linear quadratic model, \( y = \exp(-\alpha D - \beta D^2) \), where \( y \) is blood cell count expressed as fraction of pre-irradiation baseline value, \( D \) stands for radiation dose (Gray or Gy), and \( \alpha \) and \( \beta \) are the fitted coefficients for the linear and quadratic components of the linear quadratic model. The linear quadratic curve fitting was performed using SigmaPlot graphics software (SPSS Inc., Chicago, IL). RBE values were calculated by first determining the dose of \( \gamma \)-ray radiation needed to produce the same blood cell counts as proton radiation, and secondly dividing the derived \( \gamma \)-ray radiation dose by the proton radiation dose. The RBE values were plotted against proton radiation dose and analyzed by a non-linear regression analysis to show the relationship between RBE value and proton radiation dose. The non-linear regression analysis showing the relationship between the RBE value and proton radiation dose and the associated 95% confidence interval (CI) was also performed using Minitab statistical software (release 15).

RESULTS

There are no statistically significant changes in hematopoietic cell counts after confinement for 7 hours in the radiation chamber, without radiation exposure

The effect of confinement in the irradiation chamber was determined in a group of 5 animals that were placed in the irradiation chamber for up to 7 hours without radiation exposure. Using one-way ANOVA, no statistically significant differences are reported for each blood cell type (WBC, neutrophils, lymphocytes, monocytes, eosinophils, and platelets) when comparing the absolute cell count at each time point to the other time points (data not shown). Thus, the confinement up to 7 hours did not affect blood cell counts.

Radiation decreases white blood cell counts

The effects of the experimental factors, which consisted of Radiation Type, Dose, and Dose Rate, were determined by variance analyses using the general linear model. The data obtained at 3 and 48 hours after irradiation were analyzed separately to compare the effects observed at these two time points. The results showed that the WBC count decreased within 3 hours after irradiation and the magnitude of the decrease observed at 48 hours after irradiation was approximately three times the decrease observed at 3 hours after irradiation (Figure 1). At 3 hours after irradiation, Dose was a significant influencing factor of the WBC count. The Dose Rate was also a significant influencing factor of the WBC count, which resulted in larger decreased counts after irradiation at HDR than at LDR. Radiation Type (\( \gamma \)-rays vs. protons) had almost no effect on the WBC count. At 48 hours after irradiation, the Dose was the only significant influencing factor of the WBC count, which decreased in a dose-dependent manner. As shown in the dose response curves in Figure 2, a significant and dose-dependent decrease in WBC count was observed in animals at 3 hours after irradiation with HDR \( \gamma \)-rays (Figure 2A), HDR protons (Figure 2A), and LDR \( \gamma \)-rays (Figure 2B), but not with LDR protons (Figure 2B). The coefficient for the linear component of the dose response curve slope was 0.266 and 0.333 for the animals irradiated with HDR \( \gamma \)-rays and protons, which were approximately twice or more of that for the animals irradiated with LDR \( \gamma \)-rays and protons.

The coefficient for the quadratic component of the dose response curve slopes was negligible \(( \leq 3.469 \times 10^{-18} \) except for the animals irradiated with HDR \( \gamma \)-rays. The effective dose (ED) ED\(_{10}\), ED\(_{50}\), and ED\(_{90}\) for the HDR \( \gamma \)-ray radiation were bracketed by the 95% confidence intervals of the ED\(_{10}\), ED\(_{50}\), and ED\(_{90}\) for the HDR proton radiation (Table 1), indicating that all three ED values were not significantly different between the HDR \( \gamma \)-ray and HDR proton radiation. The ED\(_{10}\), ED\(_{50}\), and ED\(_{90}\) values were not compared between the LDR \( \gamma \)-ray and proton radiation due to the absence of a significant dose response for the LDR proton irradiated animals. The fitted RBE values were 2.15 and 4.02 for HDR and LDR protons at 0.75 Gy, respectively (Table 2), which were significantly above 1.00. The RBE decreased with the increase of the proton radiation dose and the fitted RBE values for HDR and LDR protons at 1 and 2 Gy were not significantly different from 1.00, which was within the 95% confidence intervals for the respective RBE values.
Figure 1. Variance analysis of white blood cell (WBC) results. Variance analysis was performed to determine which experimental factor(s) significantly affected the WBC counts in the animals 3 hours after irradiation (A) or 48 hours after radiation exposure (B). Radiation type (γ-rays vs. protons) did not significantly affect the overall WBC counts after radiation exposure whereas the dose rate did have an effect at 3 hour post-exposure, and total dose also contributed significantly to WBC count changes in the irradiated animals at both 3 hours and 48 hours after exposure. Note: the x-axes labels are included at the top of each panel, i.e., Radiation, Dose Rate, or Dose.
Figure 2. Dose-response curves for WBC in animals after irradiation. The WBC count results obtained 3 hours after irradiation at HDR (A) or LDR (B) or 48 hours after irradiation at HDR (C) or LDR (D) were determined by a non-linear regression analysis using a linear quadratic model. Statistically significant dose responses were observed for HDR and LDR γ-rays and protons at both time points except for LDR protons at 3 hours after irradiation.
Figure 3. Variance analysis of neutrophil results. Variance analysis was performed to determine which experimental factor(s) significantly affected the neutrophil count in animals 3 hours after irradiation (A) or 48 hours after radiation exposure (B). Radiation type (γ-rays vs. protons) did not significantly affect the overall neutrophil counts after radiation exposure whereas the dose rate did have an effect at 3 hour post-exposure, but not 48 hours post-exposure. Lastly, the total dose did contribute significantly to neutrophil count changes in the irradiated animals at both 3 hours and 48 hours after exposure. Note: the x-axes labels are included at the top of each panel, i.e., Radiation, Dose Rate, or Dose.
Figure 4. Dose-response curves for neutrophils in animals after irradiation. The neutrophil count results obtained 48 hours after irradiation at HDR (A) or LDR (B) were analyzed by a non-linear regression analysis using a linear quadratic model. Statistically significant dose responses were observed for HDR and LDR γ-rays and protons.
Figure 5. Variance analysis of lymphocyte results. Variance analysis was performed to determine which experimental factor(s) significantly affected the lymphocyte counts in irradiated animals 3 hours (A) or 48 hours (B) after exposure. Radiation type (γ-rays vs. protons) and dose rate did not significantly affect the overall lymphocyte count after radiation exposure, whereas the total dose contributed significantly to lymphocyte count changes in the irradiated animals. Note: the x-axes labels are included at the top of each panel, i.e., Radiation, Dose Rate, or Dose.
Figure 6. Dose-response curves for lymphocytes in animals after irradiation. The lymphocyte count results obtained from animals at 3 hours after irradiation at HDR (A) or LDR (B) or 48 hours after irradiation at HDR (C) or LDR (D) were analyzed by a non-linear regression analysis using a linear quadratic model. Statistically significant dose responses were observed for HDR and LDR γ-rays and protons at both time points. It should be noted that a common log scale is used for y-axis, which has visually exaggerated the deviation of the dose response curves from the actual data points at the high end of the radiation dose range.
Table 1. Effective doses of γ-rays and protons to decrease blood cell count by 10% (ED\(_{10}\)), 50% (ED\(_{50}\)), and 90% (ED\(_{90}\))

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose Rate</th>
<th>Time after Irradiation</th>
<th>Effective dose (and 95% CI) for γ-rays</th>
<th>Effective dose (and 95% CI) for protons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ED(_{10})</td>
<td>ED(_{50})</td>
</tr>
<tr>
<td>WBCs</td>
<td>High</td>
<td>3 hours</td>
<td>0.41 (0.14 – 0.67)</td>
<td>1.87 (0.66 – 3.10)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.91 (0.09 – 1.75)</td>
<td>6.05 (0.59 – 11.50)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>48 hours</td>
<td>0.13 (0.09 – 0.17)</td>
<td>0.81 (0.55 – 1.08)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.11 (0.07 – 0.15)</td>
<td>0.72 (0.47 – 0.96)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>High</td>
<td>48 hours</td>
<td>0.31 (0.11 – 0.52)</td>
<td>2.04 (0.71 – 3.37)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.27 (0.09 – 0.45)</td>
<td>1.79 (0.61 – 2.98)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>High</td>
<td>3 hours</td>
<td>0.05 (0.03 – 0.07)</td>
<td>0.33 (0.18 – 0.49)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.06 (0.03 – 0.09)</td>
<td>0.41 (0.21 – 0.61)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>48 hours</td>
<td>0.05 (0.02 – 0.07)</td>
<td>0.33 (0.16 – 0.49)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.06 (0.03 – 0.09)</td>
<td>0.40 (0.21 – 0.59)</td>
</tr>
</tbody>
</table>

High dose rate: 0.5 Gy/minute; low dose rate: 0.5 Gy/hour. Neutrophil counts at 3 hours post-irradiation were not decreased and not included in this table.
Table 2. Relationship between RBE and proton radiation dose for white blood cells (WBCs), neutrophils, lymphocytes.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dose Rate</th>
<th>Time after Irradiation</th>
<th>0.75 Gy</th>
<th>1 Gy</th>
<th>2 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RBE</td>
<td>95% CI</td>
<td>RBE</td>
</tr>
<tr>
<td>WBCs</td>
<td>High</td>
<td>3 hours</td>
<td>2.15</td>
<td>1.51 – 2.79</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>48 hours</td>
<td>4.02</td>
<td>2.25 – 5.79</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>48 hours</td>
<td>1.60</td>
<td>1.42 – 1.77</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>1.19</td>
<td>0.92 – 1.45</td>
<td>0.80</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>High</td>
<td>48 hours</td>
<td>1.88</td>
<td>1.31 – 2.44</td>
<td><strong>2.04</strong></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>2.11</td>
<td>1.32 – 2.91</td>
<td>0.62</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>High</td>
<td>3 hours</td>
<td>1.10</td>
<td>0.99 – 1.21</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>48 hours</td>
<td>1.41</td>
<td>1.21 – 1.61</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td></td>
<td>1.01</td>
<td>0.94 – 1.08</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td></td>
<td>0.83</td>
<td>0.67 – 1.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

High dose rate: 0.5 Gy/minute; low dose rate: 0.5 Gy/hour. Neutrophil counts at 3 hours post-irradiation were not decreased and not included in this table.
At 48 hours after irradiation, a significant and dose-dependent decrease in WBC count was observed in animals of all groups with the coefficient for the linear component of the dose response curve slope ranging from 0.786 to 1.268 (Figures 2C and 2D). The coefficient for the quadratic component of the dose response curve slopes was again negligible (≤ 1.388 x 10^{-17}) except for the animals irradiated with HDR γ-rays. The ED_{10}, ED_{50}, and ED_{90} for the HDR γ-ray radiation were greater than the upper limit of the 95% confidence intervals of the ED_{10}, ED_{50}, and ED_{90} for the HDR proton radiation (Table 1), indicating that HDR proton radiation was more effective than HDR γ-rays in reducing the number of WBCs. The ED_{10}, ED_{50}, and ED_{90} for the LDR γ-ray radiation were within the 95% confidence intervals of the three corresponding ED values for LDR proton radiation, suggesting that the LDR γ-rays and protons were not significantly different in the effectiveness of reducing the number of WBCs. The RBE values derived from results observed at 48 hours after irradiation also decreased with the increase of the proton radiation dose, but the downward trend was not as prominent as the trend observed at 3 hours after irradiation. The fitted RBE values ranged from 1.60 - 1.19 at 0.75 Gy and 1.04 - 0.77 at 2 Gy for HDR and LDR protons, respectively (Table 2), and were significantly above 1.00 for HDR protons at 0.75 and 1 Gy but significantly below 1.00 for LDR protons at 2 Gy.

**Radiation induces changes in the average neutrophil count**

Of the three experimental factors evaluated, Radiation Dose was the most significant influencing factor of the neutrophil count, which increased at 3 hours after irradiation (Figure 3A) but decreased at 48 hours after irradiation (Figure 3B), both in a dose-dependent manner. Radiation Dose Rate significantly affected the neutrophil count at 3 hours after irradiation with a larger reduction after irradiation at HDR than at LDR. The Radiation Dose Rate did not significantly affect the neutrophil count at 48 hours after irradiation. The Radiation Type did not significantly affect the neutrophil count at either time points after irradiation. At 48 hours after irradiation with HDR γ-rays, HDR protons, LDR γ-rays, and LDR protons, the coefficient for the linear component of the dose response curve slope was 0.334, 0.582, 0.386, and 0.231, respectively (Figures 4A and 4B). The coefficient for the quadratic component of the dose response curve slopes was small (≤ 0.003) except for the animals irradiated with LDR protons. The ED_{10}, ED_{50}, and ED_{90} for the HDR γ-ray radiation were greater than the upper limit of the 95% confidence intervals of the ED_{10}, ED_{50}, and ED_{90} for the HDR proton radiation (Table 1), indicating that HDR protons were significantly more effective than HDR γ-ray radiation in reducing neutrophils. The ED_{10}, ED_{50}, and ED_{90} for the LDR γ-ray radiation were bracketed by the 95% confidence intervals of the ED_{10}, ED_{50}, and ED_{90} for the LDR proton radiation, suggesting that LDR γ-rays and protons were not significantly different in their effectiveness of killing neutrophils. The fitted RBE values for irradiation with HDR protons at 0.75 and 1 Gy and with LDR protons at 0.75 Gy were 1.88, 2.04, and 2.11, respectively (Table 2), which were significantly above 1.00. The fitted RBE values for irradiation with HDR protons at 2 Gy and with LDR protons at 1 and 2 Gy were 1.55, 0.62, and 0.90, respectively, and were not significantly different from 1.00.

**Radiation decreases lymphocyte counts as early as 3 hours after radiation exposure**

Of the three experimental factors evaluated, Radiation Dose was the only significant influencing factor of the lymphocyte count at 3 hours (Figure 5A) and 48 hours (Figure 5B) after irradiation. Neither the Radiation Type nor the Dose Rate significantly affected the lymphocyte count at either time points after irradiation. The dose response curves for the lymphocytes displayed a significant and dose-dependent decrease in lymphocyte count at 3 hours and 48 hours after irradiation with either γ-rays or protons at HDR or LDR (Figure 6). The coefficient for the linear component of the dose response curve slope ranged from 1.698 to 2.148 and the coefficient for the quadratic component of the dose response curve slopes was negligible (≤ 1.180 x 10^{-16}) for all groups except the animals at 48 hours after irradiation with HDR protons and LDR protons, for which the coefficient for the linear component of the dose response curve slopes were 1.227 and 0.858 whereas the
The coefficient for the quadratic component of the dose response curve slopes were 1.159 and 0.782, respectively. The ED$_{10}$, ED$_{50}$, and ED$_{90}$ for animals at 3 and 48 hours after irradiation with HDR and LDR $\gamma$-rays were within the 95% confidence intervals of the corresponding ED$_{10}$, ED$_{50}$, and ED$_{90}$ for the HDR and LDR proton radiation (Table 1), indicating that $\gamma$-rays and protons were not significantly different in their effectiveness of reducing lymphocytes at HDR or LDR. The fitted RBE values for HDR and LDR protons at 0.75 Gy ranged from 0.83 to 1.41 at 3 and 48 hours after irradiation (Table 2). With the increase of proton radiation dose to 2 Gy, the fitted RBE values decreased to a range of 0.67 to 0.84 at 3 and 48 hours after irradiation at HDR or LDR. The RBE values were not significantly different from 1.00; however, the RBE was significantly higher than 1.00 for 0.75 Gy protons at LDR and significantly lower than 1.00 for 2 Gy HDR and LDR protons at 3 hours after irradiation and for 2 Gy LDR protons at 48 hours after irradiation.

Monocyte, eosinophil, and platelet counts were statistically insignificant at both the 3 hour and 48 hour time points (data not shown).

**DISCUSSION**

The effect of SPE-like proton radiation on hematopoietic cell counts in the ferret model was investigated in the present study. The energy of protons used to simulate SPE radiation was determined to ensure a homogenous dose distribution throughout the whole body. Hematopoietic cell loss was observed as early as 3 hours after the conclusion of radiation exposure, with a more profound decrease observed at the 48-hour time point. For the WBC and neutrophil counts determined at 3 hours after irradiation, a small but statistically significant dose rate effect was observed. Ferrets exposed to the HDR (0.5 Gy/min) radiation had lower average WBC and neutrophil counts than the animals exposed to the LDR (0.5 Gy/h) radiation. This result agrees with a similar experiment by Maks et al. (2011) in which mice were exposed to proton and $\gamma$-ray radiation at the same HDR or LDR dose rates. Although the LDR radiation exposures resulted in significant decreases in peripheral blood cell counts, the HDR radiation, for both the protons and $\gamma$-rays, resulted in even lower average blood cell counts compared to the LDR radiation. Interestingly, the dose rate effect was not observed at 48 hours after irradiation in the present study, suggesting that the higher dose rate might have only accelerated the onset of radiation effect but did not affect the overall magnitude of the radiation effect developed at the later time points. Given the fact that the dose rate effect was not observed at 48 hours after irradiation, a critical time with a pronounced reduction in circulating WBCs, neutrophils, and lymphocytes, the dose rate effect probably did not have a biologically meaningful impact on the blood cell counts in the irradiated animals.

In the present study, the time course for the changes in the neutrophil and lymphocyte counts were strikingly different. The neutrophil count displayed a dose-dependent increase at 3 hours after irradiation and a dose-dependent decrease at 48 hours after irradiation (Figure 3) whereas the dose response relationship for the lymphocytes were nearly identical between 3 hours and 48 hours after irradiation (Figure 5). It is conceivable that the increase in the neutrophil count might have partially canceled the decrease in the lymphocyte count when total WBC count was determined at 3 hours after irradiation, which might be the reason for the less pronounced dose response of the WBC count at 3 hours after irradiation. Since the neutrophils are an essential part of innate immunity, the early increase in their count could be at least partly due to rapid recruitment of these cells from the bone marrow (Christopher and Link, 2007). The different time courses for the neutrophil and lymphocyte counts observed after irradiation highlighted an importance to monitor the changes in the WBC differential in addition to the total WBC count since a change in one type of hematopoietic cells after radiation exposure may be masked by an opposite change in another type of hematopoietic cells. Based on the magnitude of the decrease and the time required to show a significant decrease in the blood cell count after irradiation, lymphocytes appeared to be the most sensitive to $\gamma$-ray and proton irradiation among the types of cells evaluated, which is consistent with the previous observation in a porcine model (Sanzari et al., 2013).

As expected, the dose of radiation was the most significant contributing factor in the
observed radiation-induced cell loss. The dose-dependent decrease in peripheral WBC counts is consistent with previous findings in the mouse model after whole-body proton (Maks et al., 2011; Ware et al., 2010; Wambi et al., 2009) and photon irradiation (Wambi et al., 2008; Pecaut et al., 2001; Gridley et al., 2001) as well as high linear energy transfer (LET) iron ion exposure (Pecaut and Gridley, 2010). The dose response relationship of proton or γ-ray irradiation on hematopoietic cell loss observed here in the ferret model represents a genetically heterogenous, non-rodent test system. The present findings suggest that damage from IR causes a significant reduction in blood cell counts in a dose-dependent manner, which may be considered a potential health risk during space travel.

The hematologic profile of ferrets has been previously determined by standard methods (Lee et al., 1982). The average leukocyte count ranges from 2.5-8.6 x 10^3 cells/μL, compared to the human total leukocyte count range of 4.5-11 x 10^3 cells/μL. While the average number of circulating leukocytes may not differ between ferrets and humans, it is worthwhile to note that the percentage of each specific type of WBC may. Therefore, when translating the results from the ferret model to human consequence, the interspecies disparity merits careful attention.

We have observed increased clotting times and the consumption of coagulation factors in ferrets exposed to the doses of SPE-like proton radiation investigated in the current report and within the same time frame (Krigsfeld et al., 2012). The consumption of coagulation factors and platelets throughout the body’s blood vessels, a condition known as disseminated intravascular coagulation (DIC), can result in internal and external hemorrhaging, and potentially death. Platelet numbers did not change in a significant manner within the first 48 hours of proton or gamma radiation exposure in this study. However, current studies underway suggest that radiation-induced coagulopathies and decreased platelet counts are present in animals exposed to the radiation doses reported in this study (up to 2 Gy) at time points later than 48 hours post-irradiation and with serious implications within weeks after radiation exposure (unpublished data). The observed coagulopathy changes in irradiated ferrets are accompanied by a severe loss of circulating white blood cells.

In conclusion, whole-body proton or γ-ray radiation results in a significant reduction in circulating WBCs in the ferret model. Using the spread-out Bragg peak to achieve a homogenous dose distribution, the proton doses used here (0.75-2.0 Gy) resulted in RBE values of 0.59 - 4.02 when evaluating radiation-induced reductions in WBC counts. Thus, whole body SPE-like radiation exposure poses threats to the hematopoietic system in an acute manner, which can result in increased susceptibility to infection, and an overall depressed immune response and function.

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Preliminary Species and Media Selection for the Veggie Space Hardware

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\textbf{ABSTRACT}

Plants will be an important component of off-Earth life support systems for food production and atmosphere recycling. “Veggie” is a small vegetable production unit designed for space flight, with a passive water delivery system. Plants can be grown in Veggie using small bags with a wicking surface containing media and fertilizer, i.e., pillows. Pillows planted with seeds can be placed on the wicking surface of the Veggie reservoir and water will wick throughout the media. Multiple small salad and herb species were grown in Veggie analog conditions using both commercial peat-based media and arcillite. Biometric measurements and microbial loads were assessed. Some species grew better in a particular media, but no general trends were apparent. Lettuce plants grew best in the blends of the peat-based and arcillite media. Microbial counts were lower on plants grown in arcillite. Four media types (peat-based mix, arcillite, and blends of the two) were tested in the rooting pillows; tests included Chinese cabbage, Swiss chard, lettuce, snow pea, and radish. Most species grew best in blends of the commercial mix and arcillite. Edible biomass production varied from 3.5-8 grams dry mass/m\textsuperscript{2}/day with lettuce having the lowest biomass and Chinese cabbage highest. Radish plants showed an increasing percentage of partitioning to edible roots with increasing arcillite in the media. Pillows appear to offer a simple, effective strategy for containing rooting media and avoiding free water while growing plants in the Veggie hardware.

\textbf{INTRODUCTION}

As we approach long-term, off-Earth human exploration, mass and volume limitations will drive the need for sustainable closed-loop life-support systems. Plants will play a critical role in these systems, both for food production and atmosphere regeneration (Wheeler et al., 2001). As a first step, a “salad machine” or vegetable production unit will serve as a technology
and generate $O_2$ (Kliss and MacElroy, 1990). The “Veggie” deployable salad-crop-production system, developed by the Orbital Technologies Corporation (ORBITEC, Madison, WI), is a modular, low mass, low energy unit with the versatility to grow a variety of crops in transit vehicles, space stations, or planetary habitat environments (Morrow et al., 2005; Morrow and Remiker, 2009; Stutte et al., 2011b). Salad crops recommended for space-life support scenarios that might be grown in a vegetable production unit such as Veggie include greens such as lettuce, spinach, chard, and mizuna, tomato, pepper, green onion, radish, herbs and strawberry (Wheeler, 2009). A Veggie unit consists of three main subsystems: an LED light cap, a bellows enclosure for the shoot environment, and a $0.14 \text{m}^2$ root mat fed by a reservoir (Morrow et al., 2005 and design revisions since). Veggie is currently considered one of the most advanced salad-machine precursors available (Stutte et al., 2009) and has passed a NASA safety review for a planned delivery and test on the International Space Station (ISS) in early 2014.

For several years, researchers at ORBITEC (Morrow and Remiker, 2009) and Kennedy Space Center (Stutte et al., 2009; Stutte et al., 2011a, 2011b) have assessed the Veggie unit for baseline salad performance under $1 \text{g}$. But a microgravity environment poses additional challenges for crop production, including maintenance of adequate nutrient and water delivery to the roots, without anoxia or salt-stress (Porterfield, 2002). A number of approaches for water/nutrient delivery have been tested for space, including porous membranes, either using constant pressure or effluent flow delivery (Wright et al., 1988; Koontz et al., 1990; Berkovich et al., 2002), porous tubes (Morrow et al., 1992; Dreschel et al., 1994), contained, vacuum operated systems (Brown et al., 1992), natural and manufactured solid substrates with sub-irrigation (Bingham et al., 1996; Goins et al., 1997), and capillary wicking from a reservoir (Kliss et al., 2000; Morrow et al., 2005; Morrow and Remiker, 2009). Active nutrient delivery provides the capability for extended cultivation scenarios by continual replenishment of water and nutrients (Goins et al., 1997), but this increased control comes with increased energy and infrastructure requirements. Passive systems, like capillary wicking, have low energy requirements, but less control of delivery under variable environmental conditions, and can only function for limited durations until nutrients are exhausted. For salad crops growing for relatively short durations, however, passive nutrient delivery can be sufficient.

Our studies focused on testing of a passive nutrient and water delivery system using rooting pillows designed for direct contact with the Veggie root mat reservoir (Stutte et al., 2011a). Pillows are small packages of growth media mixed with time release fertilizer that have a wicking surface for passive water delivery from a water conducting surface. Solid media inside pillows provides structural support for plant roots and passively distributes water and oxygen in the root zone. Seeds can be planted directly in pillows when dry, and germination can be initiated by the addition of water on orbit. Pillows are initially designed to be single use, but might be capable of growing additional crops in future scenarios. Following use, the spent pillows, which are fabricated from plastics and wicking fibers, could be compacted using devices such as a plastic melt waste compactor (Johnson et al., 2012) and used as components of building materials or to generate radiation shield panels (Wilson et al., 1997). Pillows and other disposable components can be made from polyethylene or similar materials, which are effective shields for hazardous galactic cosmic radiation (Guetersloh et al., 2006).

Our goals were to compare the growth and performance of several species and media types in rooting pillows designed for use in the Veggie unit. Species and media trials were conducted in Veggie analog conditions that provided an environment (light level, temperature, relative humidity, and water delivery) similar to what would be expected in space. Plants included small species that could be grown rapidly within the constraints of a space habitat, and consumed fresh for salad or added to packaged diets to improve flavor. Plant growth conditions, nutrition and plant species can also influence the microbial population inhabiting plant surfaces (Lindow and Brandl, 2003). The microbial load on crops
intended for consumption by astronauts must fall within an acceptable range according to NASA microbiological standards set for food. Thus, an additional aspect of these studies was to assess the microbial populations found on the plants grown using the pillow rooting system and capillary wicking.

**MATERIALS AND METHODS**

**Pillow Construction**

Pillows were constructed from 7.6 cm x 12.7 cm bags (3” x 5” re-closable static shielding bags, Uline, Pleasant Prairie, WI, external dimensions of 10 cm x 15 cm). On one side, a 5.3 cm x 10.5 cm window was cut, and a 7.5 cm x 11.5 cm piece of Nitex nylon mesh (Sefar Nytal PA-25-63, Sefar, Heiden, Switzerland) was heat welded to this surface to allow capillary wicking. Each pillow was filled with 100 mL of media and fertilizer (discussed below) and sealed using the re-closable zipper. In the non-wicking surface of the pillow, 1.5 cm slits were placed, and two 1.5 cm x 5.5 cm Nitex wicks were passed into each slit so that half of each wick protruded and the other halves were spread along the surface of the media in the pillow. Wicks were used to enclose seeds and promote early germination events by maintaining moisture around the seed.

**Trial 1: Thirteen Cultivars and Five Types of Media**

The first cultivar and media test examined 13 cultivars in eight species: lettuce (Lactuca sativa L.) cultivars ‘Outredgeous’ (Johnny’s Select Seed, Winslow, ME), ‘Flandria’ (Rijk Zwaan USA, Salinas, CA), ‘Sierra’ (Veseys, York, PEI, Canada), and ‘Oak leaf’ (Terroir Seeds, Chino Valley, AZ), mizuna (Brassica rapa cv. Nipposinica) (Seeds of Change, Santa Fe, NM), ‘Tender leaf’ vegetable amaranth (Amaranthus viridis cv. Tender leaf) (Evergreen YH Enterprises, Anaheim, CA), ‘Bright lights’ Swiss chard (Beta vulgaris var. cicla) (Terroir Seeds, Chino Valley, AZ), ‘Tokyo Bekana’ Chinese cabbage (Brassica rapa, Chinensis group) (Evergreen YH Enterprises, Anaheim, CA), ‘Sugar Pod II’ snow pea (Pisum sativum cv. Sugar Pod II) (Evergreen YH Enterprises, Anaheim, CA), ‘Spicy Globe’ basil (Ocimum basilicum minimum ‘Spicy Globe’) (Ferry-Morse Seed Co., Fulton, KY), ‘Genovese’ basil (Ocimum basilicum ‘Genovese’) (Terroir Seeds, Chino Valley, AZ), common chives (Allium schoenoprasum) (Veseys, York, PEI, Canada), and Greek oregano (Origanum heracleoticum) (Terroir Seeds, Chino Valley, AZ).

All of the leafy greens selected can be grown using the cut-and-come-again strategy, essentially repeated harvests from the same plant, a harvest scenario that will potentially be employed in Veggie. Lettuce cultivars ‘Flandria’ and ‘Outredgeous’ have been previously studied for growth in off-Earth scenarios (Richards et al., 2006; Stutte et al., 2009; Stutte et al., 2011a, b). ‘Sugar Pod II’ snow pea was selected to test a small reproductive species in the pillows. The herbs were chosen to provide different growth habits and with the intent that they could easily be used to add interest and variety to a diet based on pre-packaged thermally stabilized foods.

These 13 cultivars were tested in both Fafard #2, a peat-based commercial potting mix also containing perlite and vermiculite, (Conrad Fafard Inc., Agawam, MA) and in arcillite (sifted 1-2mm, Turface Proleague, Profile LLC, Buffalo Grove IL) using the rooting pillows. The 1-2 mm arcillite medium has been well studied for use in microgravity (Jones et al., 2002; Norikane et al., 2005). Additionally, two cultivars, ‘Outredgeous’ and ‘Sierra’ lettuce were tested with five media types: Fafard #2, arcillite (1-2mm), a 1:1 blend of the two media, a 7:3 blend (70% Fafard #2: 30% arcillite) and a 1:1 blend of coarse perlite and fine vermiculite (PVP Industries, North Bloomfield, OH). Media were autoclaved and mixed with Nutricote (18-6-8, type 180, Florikan, Sarasota, FL) slow release fertilizer at a rate of 7.5 g/L dry media. Pillows were weighed and then soaked in de-ionized (DI) H₂O for five minutes prior to planting.

Fresh media was soaked in DI H₂O for 70 hours to check pH levels. Starting pH of the media with fertilizer ranged from 5.76 for the Fafard #2 to 6.56 for the arcillite with the blends having intermediate values. The perlite:vermiculite blend was higher at 6.64. Two slit openings were planted in each pillow except for chives, where ten openings were planted per pillow. Two seeds were planted per opening and thinned to one plant per opening upon germination and emergence, except in the case of
snow pea where one seed per opening was planted due to large seed sizes. Pillows were placed Nitex-side-down in Veggie reservoir analogs, which consisted of polypropylene sterilizing pans (14.2 L, Thermo Fisher Scientific, Rochester NY) with a sheet of soft foam (43.5 cm x 35.5 cm x 5 cm, Uline, Pleasant Prairie, WI), topped with a 420 mm x 340 mm x 2 mm sheet of PVC and wrapped with a 680 mm x 520 mm piece of Nomex wicking cloth (Aramid fabric, InsulSafe Textiles). PVC was placed over the foam to maintain uniform moisture and eliminate the impact of air bubbles under the cloth. Thirteen pillows were placed in each tub with cultivars randomized in three tubs (two with Fafard #2, one with arcillite). Thirteen pillows of ‘Outredgeous’ or ‘Sierra’ lettuce were placed in each of two other tubs with five media types randomized.

Initially, 2 L of DI H\textsubscript{2}O were placed in each tub. Water use was monitored and the level was adjusted daily with DI H\textsubscript{2}O to maintain adequate water in the tubs. Tubs were covered with plastic wrap to increase internal humidity until ¾ of the seeds had germinated. Plants were grown under 16-h photoperiods with initially 150 µmol·m\textsuperscript{-2}·s\textsuperscript{-1} triphosphor fluorescent light (Sylvania FP541/841/HO). Light levels were increased to 300 µmol·m\textsuperscript{-2}·s\textsuperscript{-1} at 9 days after planting (DAP). Seeds that failed to germinate were replanted within 5 DAP. Plants were grown at 24°C for the first week and then the temperature was ramped to 28°C day / 24°C night by 9 DAP. Relative humidity was adjusted to 70% day / 75% night and CO\textsubscript{2} was set to 1200 µmol mol\textsuperscript{-1} so that environmental conditions were relevant to those measured in the Veggie hardware with bellows closed and lights cycling in a high CO\textsubscript{2} environment like the ISS. All plants were harvested at 36 DAP and root-filled pillows were weighed and characterized at 37 DAP.

Upon harvest, roughly one third of the plants were reserved for microbial load analysis. Plants were photographed and biometric analysis included measurements of chlorophyll, leaf area, fresh and dry mass. Chlorophyll measurements were obtained by averaging the SPAD values of three non-senescent leaves per plant (SPAD-502, Konica Minolta, Osaka, Japan), and average values per pillow were calculated from the two plants. Leaf area was only measured on plants not used for microbial analysis. Shoot tissues were bagged and dried in an oven at 70°C for greater than 72 h. Oven dry tissue was allowed to equilibrate to room temperature and RH for an hour prior to weighing.

Qualitative measurements of root status were obtained by observing pillow fill with roots through the translucent Nitex surface using the following criteria: Pillows filled to excess with roots bulging and solid mats of roots were considered root bound. Pillows ¾ full to full but without bulging were considered full. Pillows ½ to ¾ full were considered half full. Pillows less than ½ full were considered low. Pillows were also weighed and dried, and dry mass was obtained, however pillow-to-pillow media variability and the relative mass of roots to media made it difficult to estimate root dry mass. Arcillite is significantly heavier than dried root tissue, while peat, perlite and vermiculite are similar in mass to dried root tissue.

**Microbial Load Analysis**

Plants used for microbial analysis were from one set of all cultivars grown in Fafard #2 and one set each of ‘Outredgeous’ and ‘Sierra’ lettuce grown in three types of rooting media: Fafard #2, 1:1 blend of Perlite and Vermiculite, and arcillite (1-2 mm), described above. The mixes of Fafard #2 and arcillite were not analyzed for microbial load. The two plants grown in one pillow were poolsed samples. Shoot tissue was cut from the plants and weighed using aseptic technique and placed into sterile blender bags (BagLight, Interscience Labs, Weymouth, MA). Sterile DI H\textsubscript{2}O was added to each sample in a 1:10 weight/volume ratio. Bags containing sample and diluent were placed in a bag mixer (Interscience Labs, Weymouth, MA) and blended for 2 minutes to remove microbes from plant surfaces. The sample extracts were serially diluted in sterile DI H\textsubscript{2}O and plated in duplicate onto Difco R2A agar (BD, Franklin lakes, NJ). Plates were incubated at 28°C for 48 hours before enumeration of colonies to determine colony-forming units (CFU) per gram of tissue. Microbial sampling and analysis methods were modified from Pouch Downs and Ito (2001).

**Trial 2: Five Cultivars in Four Types of Media**

Five cultivars from four plant families were tested in the second trial. Cultivars were ‘Cherry

Four previously used media were tested: Fafard #2, arcillite (1-2 mm), and 7:3 and 1:1 mixtures of the two of these. Pillows and planting were as described for Trial 1. Sixty pillows were grown in five tubs, therefore there were twelve pillows of each cultivar with three pillows of each media type per cultivar. The sixty pillows were completely randomized within the five tubs, giving twelve pillows per tub arranged in alternating orientation within the tubs to provide maximum spacing for each plant and reduce shading.

Planting and experimental design followed the procedures described above, except that temperature was ramped up to 28°C by 7 DAP and light was maintained at 200 μmol·m⁻²·s⁻¹ for the duration of the experiment. Plants were harvested at 28 DAP and root-filled pillows were characterized and weighed at 29 DAP. Harvest measurements were similar to those described above except that microbial analyses were not performed. Also, plant height and qualitative status were noted but leaf area measurements were not performed. Photographs and measurements of chlorophyll, fresh mass, dry mass, pillow status, and pillow mass were all taken as previously described.

Statistical Analysis

Data are presented on a per pillow basis. Data were analyzed using GraphPad Prism® software with reference to Motulsky, 2003. Analysis of variance (ANOVA) was performed on data for chlorophyll, leaf area, height, fresh mass, dry mass, and biomass partitioning (radish). Data are presented as true means. Mean separation was performed by Bonferroni post-tests for two-way ANOVA.

RESULTS AND DISCUSSION

Two plants, (except for chives), were allowed to grow in each pillow as shown in Figure 1, however seeds did not always germinate simultaneously, and light interception often gave the earlier plant a competitive advantage. For this reason, data are discussed on a per-pillow basis rather than on a per-plant basis. Chlorophyll and height measurements are averages of both plants in a pillow, while leaf area, fresh mass, and dry mass are totaled per pillow.

Figure 1. Examples of salad crops growing in pillows. A. ‘Outredgeous’ lettuce in 1:1 Fafard #2: arcillite and B. ‘Cherry Bomb II’ radish in arcillite at harvest of trial 2 (28 DAP). Scale bars are 10 cm.
Trial 1A: Thirteen Cultivars in Two Types of Media

For the broad survey of thirteen species in two types of media, media was not found to be a significant factor in any of the parameters measured across species. Across media, differences between species were found to account for large percentages of the variation in chlorophyll content (59%), leaf area (78%), fresh mass (48%) and dry mass (80%). Greek oregano seeds failed to germinate in pillows with either of the media used, so only twelve species were analyzed, and due to the diversity of chlorophyll levels, size, and growth rate of these species, no general response to media effects can be assessed. Media response was observed in some species, with, for example, delayed growth in a particular type of media, however small sample sizes limit conclusions. Figure 2 shows the dry mass per pillow of the twelve species tested in two media types.

Figure 2. Shoot dry mass of twelve species grown in pillows in two different media. Data are per pillow and a pillow contains two plants (except chives which had 10 plants per pillow). Mass is the sum of the shoot dry mass of all plants in a pillow. Each bar represents one pillow.

Cultivars with favorable growth in pillows include ‘Tokyo Bekana’ Chinese cabbage, ‘Sugar Pod II’ snow pea, mizuna, ‘Bright Lights’ Swiss chard, ‘Spicy Globe’ basil and the lettuce varieties ‘Outredgeous’ and ‘Sierra’, based on biomass accumulation and observations of plant health.

Trial 1B: Two Species in Five Types of Media

Two cultivars of lettuce, ‘Outredgeous’ and ‘Sierra’, were tested for growth in five types of media. Both grew well in pillows and showed no significant variation in leaf area due to different media types. ‘Sierra’ lettuce showed a chlorophyll response to media, with plants growing in perlite: vermiculite having significantly higher SPAD readings than plants growing in arcillite (P<0.05), but no chlorophyll response was observed in ‘Outredgeous’. Fresh and dry mass were significantly different depending on media type. Plants growing in mixtures of Fafard #2 and arcillite had greater mass than those grown in other media, as shown in Figure 3, and in both cultivars, plants grown in 7:3 and 1:1 mixtures of Fafard #2: arcillite showed significantly greater fresh (not shown) and dry mass accumulation than those grown in the 1:1 mixture of perlite: vermiculite. Trends for the observed percentage of root fill in the pillows mirrored those for dry mass (data not shown), with percentages varying from half to full in Fafard #2 and perlite: vermiculite, and the Fafard: arcillite blends being mostly root-bound.

Trial 1: Microbial Load Analysis

Currently the limit for aerobic bacteria on a non-thermostabilized food item is ≤ 2 x 10⁴ colony forming units (CFU) per gram (Perchonok and Douglas, 2012). Studies done to determine the microbial numbers in field and retail samples of a variety of leafy green produce items report total aerobic bacteria levels in the range of 10⁴ to 10⁷ CFU/gram (Johnston et al., 2005; Ruiz et al., 1987). Our analysis of twelve cultivars of leafy greens and herbs indicates microbial densities (2.2 x 10⁵ to 1.8 x 10⁶) consistent with these studies with the exception of basils, Chinese cabbage, and lettuces grown in arcillite, in which bacterial counts were less than 10⁴/gram. Higher numbers of aerobic bacteria on produce are not necessarily correlated with decreased shelf life, quality or an indication of pathogen contamination (Johnston et al., 2005).
Figure 3. Shoot dry mass of lettuce grown in five different media types. Data are means of two pillows per media type for each cultivar, with each pillow containing two plants. Error bars indicate standard deviations. Means within a cultivar with different letters are significantly different at $P<0.05$.

For the cultivars grown in Fafard #2, counts ranged from $1.9 \times 10^3$ on snow pea to the highest bacterial count, $1.8 \times 10^6$, on mizuna. Microbial analysis was also performed on one pillow of each ‘Sierra’ and ‘Outredgeous’ lettuce grown on three media types: Fafard #2, arcillite, and 1:1 perlite: vermiculite. Lettuce plants grown in arcillite exhibited aerobic plate counts approximately 1-2 orders of magnitude lower than those grown in perlite: vermiculite and Fafard #2. This information is important in the selection of potential food crops and growth media when considering microbiological standards, however further analysis with greater numbers of replicates needs to be performed, and the more-productive media blends were not tested. Additionally, seeds were not sterilized prior to these tests, and sterilization might lead to reduced microbial levels on produce. Many of the microorganisms detected on the leaves are likely brought in through air flow and human tending, however, so produce sanitation using a sanitizing agent safe for spaceflight is one option to reduce microbial loads in crops and conditions where they exceed allowances.

**Trial 2: Five Species in Four Types of Media**

Several of the highly productive species observed in trial 1 were selected for further study in trial 2. Radish, a crop well-studied for use in space (Goins et al., 2003; Richards et al., 2006) was added for comparison. Due to the media response seen among lettuce cultivars in trial 1, the perlite: vermiculite blend was dropped from consideration and only four media types were examined: Fafard #2, arcillite (1-2 mm), and 7:3 and 1:1 mixtures of the two media. Of the parameters measured, average plant height showed no media dependency (data not shown). Plants grown in pillows reached the same average height at harvest regardless of growth media, indicating that height in these species may be predetermined and not responsive to root zone environments that affect other aspects of plant growth.

Interestingly, qualitative observations of root fill in the pillows also were species-dependent with only slight media differences (data not shown). Snow pea pillows were root bound in all media types, while radish pillows were less than half-full of fibrous roots in all media. Other species were intermediate, with Swiss chard pillows full to root bound, and Chinese cabbage and lettuce pillows half to full. These three species generally showed an increase in roots with increasing levels of arcillite (data not shown).

Chlorophyll content was affected by the type of species and to a lesser extent the type of media. Figure 4 shows that the SPAD readings of radish, snow pea, and chard were not different from one another and averaged in the 40-50 range. Chinese cabbage and lettuce both had lower chlorophyll contents (SPAD~15-30) and were not different from each other regardless of media (Fig. 4). Some media effects were observed, but generally chlorophyll contents (SPAD data) were much more species dependent than media dependent, with species accounting for 80% of the observed variation and media only accounting for 3%. This response is consistent with sufficient macro/micronutrients being available from the slow release fertilizer in the media.

In contrast to the slight impact of media type on chlorophyll levels, fresh mass was dependent on both media type and species, with species accounting for 33% of the observed variation and...
media accounting for 28%. Interaction between species and media was not significant. Radish had the greatest fresh mass of all species tested, as seen in Figure 5. For media, Fafard#2 consistently produced plants of all species with the lowest fresh mass, and the Fafard #2: arcillite mixtures tended to produce larger plants, likely related to the balance between moisture holding capacity and aeration of the media.

**Figure 4.** Average chlorophyll content for five species grown in four types of media. Data are averages for three pillows, with pillow values the average of two plants, and plant values averaging three leaves on a plant. Means within plant species between media types with different letters are significantly different at $P<0.05$. Where letters are absent there is no significant difference within a species. Error bars indicate standard deviations.

Dry mass data displayed an interaction between species and media that was not observed in fresh mass or other parameters, documented in Table 1. Swiss chard, lettuce and snow pea showed no significant response to media and produced the same dry mass as each other. Chinese cabbage plants however, produced a significantly greater dry mass in 1:1 Fafard #2: arcillite than in any of the media tested ($P<0.05$). The dry mass of Chinese cabbage produced in 1:1 Fafard #2: arcillite was significantly greater than any other species in the same media ($P<0.01$). Also, Chinese cabbage produced significantly more dry mass than lettuce in every media tested ($P<0.05$), however there were no differences between the two in fresh mass (see Fig. 5) suggesting a high quantity of water in lettuce compared to the cabbage. Radish plants produced significantly more dry mass in 7:3 Fafard #2: arcillite than in any of the other media types ($P<0.05$), and since radish showed excellent growth in the 7:3 mixture, a significantly greater dry mass was produced in this media by radish than by either Swiss chard, lettuce or snow pea ($P<0.01$), and a slightly greater dry mass than Chinese cabbage (Table 1).

**Figure 5.** Shoot fresh mass of five species grown in four types of media. Data are averages for three pillows, with pillow values the sum of the shoot (shoot and tap root for radish) fresh mass of two plants. Means within plant species between media types with different letters are significantly different at $P<0.05$. Where letters are absent there is no significant difference within a species. Error bars indicate standard deviations.

General trends indicate that Chinese cabbage and radish dry mass accumulation responds to media more than other species. Although similar in fresh mass, Chinese cabbage generally produces the greatest dry mass, while lettuce the
least of any of the crops tested. Radish produced significantly greater dry mass than most other species in the 7:3 mixture, however, these data are total biomass. When only the tap root is examined this value falls to 1.95 g dry mass on average. Table 2 provides an estimate of the maximum growth rate of each of these species. These edible growth rates are comparable to those for lettuce and soybean observed by Wheeler et al., 2003, though they are lower than for other staples such as potato.

Table 1. Average shoot dry mass (g) (shoot and tap root for radish) per pillow of five species grown in four media types. Data are averages for three pillows, with pillow values the sum of the dry mass of two plants. Means between plant species within a media type with different letters are significantly different at P<0.05. Asterisks indicate means within a species that are significantly different between media types (P<0.05)

<table>
<thead>
<tr>
<th>Plant</th>
<th>MEDIA TYPE</th>
<th>Fafard #2</th>
<th>7:3 Fafard: Arcillite</th>
<th>1:1 Fafard: Arcillite</th>
<th>Arcillite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabbage</td>
<td>2.21 A</td>
<td>2.29 AB</td>
<td>3.36 A*</td>
<td>2.21 A</td>
<td></td>
</tr>
<tr>
<td>Chard</td>
<td>0.92 B</td>
<td>1.86 BC</td>
<td>1.78 B</td>
<td>1.55 AB</td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>0.78 B</td>
<td>1.24 C</td>
<td>1.46 B</td>
<td>1.07 B</td>
<td></td>
</tr>
<tr>
<td>Radish</td>
<td>1.63 AB</td>
<td>3.10 A*</td>
<td>1.93 B</td>
<td>1.61 AB</td>
<td></td>
</tr>
<tr>
<td>Snow pea</td>
<td>1.25 B</td>
<td>1.53 BC</td>
<td>1.74 B</td>
<td>1.84 AB</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Calculated maximum edible growth rates of tested species in highest yielding media (highest yielding for dry mass). Calculations use a 28 day growth period and a pillow with external area of 0.015 m². Mass is an average of three pillows with two plants per pillow. Radish data are for tap root mass while other data are for shoot mass.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Edible Growth Rate</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g FM/m²/day)</td>
<td>(g DM/m²/day)</td>
</tr>
<tr>
<td>Cabbage</td>
<td>42.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Chard</td>
<td>24.3</td>
<td>4.4</td>
</tr>
<tr>
<td>Lettuce</td>
<td>38.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Radish</td>
<td>44.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Snow pea</td>
<td>21.8</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Interestingly, although total fresh mass of radish demonstrated a significant influence of media, individually, there was no significant difference between shoots grown in different media types or roots grown in different media types. Figure 6 shows that dry mass, however, was significantly different, with plants grown in 7:3 Fafard: arcillite having significantly greater shoot mass than arcillite- or 1:1-grown plants and significantly greater root mass than Fafard #2-grown plants. When the percentage of biomass in the root and shoot are calculated we observe increasing partitioning into the edible root with increasing concentration of arcillite and decreasing percentage of Fafard #2 in the media, as shown in
Figure 7, and the interaction between media and the location of partitioning is highly significant (P<0.0001).

Figure 6. Dry mass of radish shoots and edible tap roots when grown in four types of media. Data are averages for three pillows, with pillow values the sum of two plants. Error bars indicate standard deviations. Means with different letters are significantly different at P<0.05.

Figure 7. Biomass partitioning between the storage root and shoot calculated from dry mass. Data are means from three pillows (six plants) of each media types and error bars indicate standard deviations.

The observed differences in response to media may be due to a variety of media characteristics including moisture, aeration, and cation and anion exchange capacity, however since water is not leaching through the pillows it seems likely that water and aeration may be playing the largest role. The arcillite, a porous clay, has a lower water holding capacity (~50% by weight) than the peat-based Fafard #2 medium which expands when wet and can hold ~60% moisture by weight. The physical and chemical parameters of the media can lead to differences in root growth and nutrient availability, and several of these parameters are gravity-dependent. Future testing will further assess these challenges; however initial results using the pillow concept indicate that this technique may be a good approach to growth of salad and herb crops for the Veggie hardware.

CONCLUSIONS

The Veggie space hardware is designed to grow small salad crops using passive water distribution. Pillows, small packets of media and time-release fertilizer, were designed to interface with this hardware to supply water, nutrients, and structural support for crop growth. Species varied in their growth in pillows, however most salad greens and small herbs tested grew acceptably in these confined volumes. Some species had media-dependent growth, but certain parameters, such as plant height and chlorophyll level, did not vary much with media type. Shoot microbial loads were generally lower in plants grown in arcillite than in other types of media tested. A mixture of Fafard #2 and arcillite produced larger lettuce plants than these media separately, while lettuce did not grow well in a perlite: vermiculite combination. Chinese cabbage and radish had a greater dry mass response to media than other species tested, and both did best in blends of Fafard #2: arcillite though not at the same ratios. Radish plants showed increased carbon partitioning to the root zone and decreased partitioning to the shoot with increasing levels of arcillite in the media. Although a number of media characteristics may influence the growth of these species, it seems likely that water and aeration may be the most important parameters for these small pillows, and the impact of...
Massa et al. -- Species and Media Selection for Veggie

Gravitational and Space Research Volume 1 (1) Oct 2013 -- 105

microgravity on these characteristics will need to be determined. Small, single-use pillows appear to provide acceptable growth of a variety of salad and herb species for the Veggie spaceflight hardware.

REFERENCES


Massa et al. -- Species and Media Selection for Veggie

Automotive Engineers (SAE) Technical Paper 2005-01-2843.


April E. Ronca\textsuperscript{1,2}, Joshua S. Alwood\textsuperscript{1}, Ruth K. Globus\textsuperscript{1}, and Kenneth A. Souza\textsuperscript{3}

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ABSTRACT

The \textit{Mark III Rodent Habitat Workshop} was held at NASA Ames Research Center on March 21-22, 2013 to prepare top-level science requirements for developing a habitat to support studies of mammalian reproduction and development on the International Space Station (ISS). This timely workshop assembled a diverse team with expertise in reproductive and developmental biology, behavior, space biosciences, habitat development, physiology, mouse genetics, veterinary medicine, rodent husbandry, flight hardware development (rodent), and spaceflight operations. Participants received overview presentations from each discipline, discussed concerns, potential risks, and risk mitigations corresponding to distinctive reproductive and developmental phases, and reviewed specific examples of research within the major space bioscience disciplines requiring a Mark III habitat\textsuperscript{1} to achieve their objectives. In this review, we present the workshop materials and products, and summarize major recommendations for defining the requirements envelope for the NASA Rodent Habitat (RH) Mark III. Development of this habitat will permit the first long duration studies of mammalian reproduction and development in space, within and across generations.

INTRODUCTION

Over the past 25 years, four independent advisory boards have been convened by the National Research Council (NRC) (1987, 1991, 1998, 2011) to guide the future of NASA’s Space Biology research. The corresponding reports have uniformly emphasized the fundamental importance of research on reproduction and development of mammals in space, and

\textsuperscript{1}Mark III Rodent Habitat refers to the sequential habitat development effort beginning with the Rodent Habitat for ISS, Mark I, scheduled to fly adult rodents in April 2014.
specifically that animals be studied within and across generations, completing at least two complete life cycles in space. The recent Decadal Survey published in 2011, *Recapturing a Future for Space Exploration: Life and Physical Sciences Research for a New Era*, ranks reproductive and developmental biology research and generational studies of rodents within the space environment among the highest priority NASA initiatives.

Spaceflight studies on rodent reproduction and development have revealed important, yet preliminary, findings and “lessons learned” (Maese and Ostrach, 2002; Ronca, 2003). Special requirements of reproducing and developing animals, restrictions on experimental design due to hardware limitations, short duration exposures to the space environment precluding analysis across multiple reproductive cycles or significant periods of development, have not permitted a comprehensive approach to life-cycle issues (Moody and Golden, 2000; NRC Report 2011). As ISS research capabilities expand to support studies of adult rodents (Cancedda et al., 2012; Mark I flight), there is an opportunity and an expectation that well-designed and highly controlled studies of reproducing and developing animals will follow, addressing the recommendations of advisory panels. Such experiments will be of significant interest to the reproductive and developmental biology research communities and offer unprecedented opportunities to study developing and adult animals and specimens never exposed to Earth gravity. Finally, reproductive and developmental biology research using a mammalian model holds immense promise for advancing translational knowledge with major relevance to current and future space habitation by humans.

The major goal of the Rodent Mark III Habitat Workshop was to identify the top-level science requirements envelope suitable for meeting the research objectives of the science community. This is the first animal habitat specifically designed to support varying stages of reproduction and development of rodents for research on the ISS. Precedence is given to studies of rats and mice examining transmission across generations of structural and functional changes induced by exposure to the space environment. This directive encompasses key reproductive and developmental phases comprising the mammalian life cycle. There was strong consensus among workshop participants that, to attain this goal, spaceflight animal housing and hardware will need to support multiple neural, endocrine, and environmental requirements to maximize successful outcomes for: (1) mating, (2) conception, (3) pregnancy, (4) embryonic/fetal development, (5) birth, (6) lactation, (7) maternal care, and (8) offspring development through sexual maturity. Achieving these individual milestones and their repeating cycle will form the foundation for lifespan and multigenerational research success.

WORKSHOP CHARTER AND OVERVIEW

The Rodent Mark III Habitat Workshop was Co-Organized and Co-Chaired by Ken Souza and April Ronca. Josh Alwood served as the Executive Secretary, and Ruth Globus is the ISS Rodent Habitat Project Scientist. Approximately 20 participants met for two days at the Ames Research Center. Participants included experts in animal husbandry (rodents), animal behavior, reproductive and developmental biology, animal physiology, veterinary medicine, flight hardware development, and spaceflight operations (Table 1, Workshop Participants and Affiliations). In preparation for the workshop, participants were asked to review the NASA Rodent Research Science Requirements Envelope Document (SRED), Revision E, appropriate sections of the NRC’s recent Decadal Survey of the Life and Physical Sciences (2011), and other relevant material.

During the workshop the participants: (1) Received an overview briefing covering the characteristics of the ISS Rodent Habitat (a.k.a., Animal Enclosure Module (AEM-X)) that is under development for flight in April 2014; (2) Considered and discussed what is known about rodent reproduction and development in space; (3) Discussed the current requirements and capabilities of the rodent habitat for transporting animals to the ISS, Animal Enclosure Module–Transfer (AEM-T), and the on-orbit habitat, AEM-X, and any additional or expanded requirements specific to the mating, birthing, nursing, and maturation of rodents on the ISS; (4) Determined any new research that is required to close gaps in knowledge needed to define the
Table 1. List of Participants

<table>
<thead>
<tr>
<th>Participant</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeffrey Alberts, PhD (In absentia)</td>
<td>Indiana University, Bloomington</td>
</tr>
<tr>
<td>Joshua S. Alwood, PhD Executive Secretary</td>
<td>Oak Ridge Associated Universities</td>
</tr>
<tr>
<td>Ted A. Bateman, PhD</td>
<td>University of North Carolina</td>
</tr>
<tr>
<td>Shawn G. Bengtson</td>
<td>McGowan Institute, University of Pittsburgh</td>
</tr>
<tr>
<td>Allison Brown</td>
<td>LifeSource Biomedical, LLC</td>
</tr>
<tr>
<td>Kristin D. Evans, PhD, DVM</td>
<td>University of California, Davis</td>
</tr>
<tr>
<td>Charles A. Fuller, PhD</td>
<td>University of California, Davis</td>
</tr>
<tr>
<td>Ruth K. Globus, PhD</td>
<td>NASA Lead, NASA Ames Research Center</td>
</tr>
<tr>
<td>Mike Hines</td>
<td>NASA Ames Research Center</td>
</tr>
<tr>
<td>Danny A. Riley, PhD</td>
<td>Medical College of Wisconsin</td>
</tr>
<tr>
<td>April E. Ronca, PhD, Workshop Co-Chair</td>
<td>Wake Forest School of Medicine</td>
</tr>
<tr>
<td>Stephanie Solis, DVM</td>
<td>LifeSource Biomedical, LLC</td>
</tr>
<tr>
<td>Kenneth A. Souza, Workshop Co-Chair</td>
<td>Logyx, LLC</td>
</tr>
<tr>
<td>Marianne Steele, PhD</td>
<td>Lockheed Martin</td>
</tr>
<tr>
<td>Louis S. Stodieck, PhD</td>
<td>BioServe Space Technologies, University of Colorado at Boulder</td>
</tr>
<tr>
<td>Joseph S. Tash, PhD</td>
<td>University of Kansas Medical Center</td>
</tr>
</tbody>
</table>

requirements necessary for habitat development; (5) Identified specific examples of developmental and reproductive research on the ISS that require a RH Mark III habitat; and (6) Observed the rodent habitat (AEM) flown on the Space Shuttle: the nursing insert for the AEM flown on the Space Transportation System (STS) STS-72 NIH-R3 mission in 1996 and a prototype Animal Development Habitat developed by STAR Enterprises, Inc. as a Small Business Innovative Research (SBIR) project in 1986.

Workshop products and follow-on activities include a detailed report, viewable at URL: http://spacebiosciences.arc.nasa.gov/media/Mark3_v1.14_signed_rotated.pdf, that includes: (1) A summary of the discussions and recommendations of workshop participants, (2) Specific top-level science requirements suitable for defining the envelope for the RH Mark III habitat, (3) Recommendations for ground-based research needed to close gaps in knowledge where appropriate requirements cannot be specified, and (4) Examples of flight experiments that are consistent with the 2011 NRC Decadal Survey recommendations and would require a Mark III Rodent habitat to achieve their objectives.

Specifically, the workshop report will be used to expand the current ISS Rodent Research SRED (Revision E) to include the requirements for Reproductive and Developmental Biology. In addition, it will provide the guidance necessary for a RH Mark III project team to develop more...
detailed science requirements and an engineering specification for the development of the RH Mark III habitat.

**SCIENCE TEAM PRESENTATIONS FOR RESEARCH USING A RH MARK III HABITAT**

Prior to the workshop, participants were organized into five different science teams (Reproduction, Neuroscience and Behavior, Musculoskeletal, General Physiology and Immunology, and Commercial Interests). Each team was tasked with identifying key research hypotheses and problems requiring a RH Mark III habitat, and incorporating key phases of mammalian reproduction and development (Figure 1). Team leaders presented the findings (Table 2).

**Figure 1. Multigenerational Success is a Repeating Cycle of Necessary Milestones (Courtesy of Joseph Tash, Kansas University Medical Center).**

**WORKSHOP PRESENTATION SUMMARIES**

**Historical Perspective – A. Ronca**

Mammalian development has been studied in seven nascent, yet pioneering, spaceflight missions. To determine whether mammals can conceive in microgravity, the unmanned biosatellite Cosmos 1129, flown in 1979, provided rats the opportunity to mate in flight. However, no pregnancies were realized at recovery (Keefe, 1985; See Tash, Reproductive Biology, this review). In 1983, Cosmos 1514, led by Luba Serova, Dick Keefe, and Jeff Alberts, carried late...
Table 2. Examples of relevant flight experiments requiring a RH Mark III Habitat.

<table>
<thead>
<tr>
<th>Reproductive Biology – Lead: J. Tash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multigenerational survival comprises a cycle of lifespan functional milestones at the multiple levels: Whole animal, organ and endocrine systems, and cell</td>
</tr>
<tr>
<td>• A failure or deficit in any milestone compromises species survival</td>
</tr>
<tr>
<td>Does male and female sexual definition and development proceed normally in the space environment?</td>
</tr>
<tr>
<td>• Gonad development, maturation and health</td>
</tr>
<tr>
<td>• Gamete production, maturation and health</td>
</tr>
<tr>
<td>Are patterns of social behavior and mating affected by the space environment?</td>
</tr>
<tr>
<td>• Ex-gonad gamete maturation in the female reproductive tract</td>
</tr>
<tr>
<td>• Fertilization, conceptus, placenta, fetal development</td>
</tr>
<tr>
<td>• Support of pregnancy, birth, lactation, nursing, weaning</td>
</tr>
<tr>
<td>• Post-weaning growth, puberty, acquisition of sexual maturity</td>
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<table>
<thead>
<tr>
<th>Neuroscience and Behavior – Lead: A. Ronca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the neural architecture of the brain, particularly the gravity sensing system, shaped by gravity?</td>
</tr>
<tr>
<td>• Morphology of the neurovestibular system</td>
</tr>
<tr>
<td>Are vestibular mediated behaviors shaped by gravity, and are these correlated with changes in vestibular morphology?</td>
</tr>
<tr>
<td>Is development of the motor system dependent upon gravitational input?</td>
</tr>
<tr>
<td>• Emergence of fine motor control of locomotion and gait may require gravitational input during development</td>
</tr>
<tr>
<td>Are there critical periods during pre- and/or postnatal life during which gravity exerts formative effects?</td>
</tr>
<tr>
<td>Does lack of gravitational input to the vestibular macular sensory organs, beginning prior to conception and continuing into adulthood, ‘developmentally program’ circadian and homeostatic processes across the lifespan and generations?</td>
</tr>
<tr>
<td>Is there epigenetic (non-genomic) cross-generational heritability of early life programming by gravity?</td>
</tr>
<tr>
<td>• Identifiable epigenetic changes in DNA methylation patterns may be associated with development in microgravity (a direct effect) distinct from indirect maternal contributions to epigenetic programming of offspring phenotype</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Musculoskeletal – Lead: D. Riley</th>
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</thead>
<tbody>
<tr>
<td>For animals born in space and undergoing development, growth and aging are there critical periods of development and growth that require gravity?</td>
</tr>
<tr>
<td>• On-orbit, temporal, noninvasive bone density and shape measurements, body weight to assess growth, temporal muscle diameter and length measurements, tissue acquisition and preservation of bone, cartilage, and muscle at key time points. Intact sample return for earth-based analyses</td>
</tr>
<tr>
<td>Is stem cell production (myoblasts, osteoblasts, osteoclasts, bone marrow cells) reduced during prolonged spaceflight?</td>
</tr>
<tr>
<td>• Quantify stem cell 1G to evaluate reloading injury and the capacity for repair that is dependent on stem cell participation. 1G restores stem cell deficiency or reveals permanent deficiencies</td>
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<thead>
<tr>
<th>General Physiology &amp; Immunology – Lead: C. Fuller</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Physiology &amp; Immunology</td>
</tr>
<tr>
<td>• Altered regulation (System to Genome)</td>
</tr>
<tr>
<td>• Adaptive capabilities (i.e., 0-&gt;1G)</td>
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</tbody>
</table>
Table 2. Examples of relevant flight experiments requiring a RH Mark III Habitat (continued).

<table>
<thead>
<tr>
<th>CNS Function</th>
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<tbody>
<tr>
<td>• Covered by Neuroscience &amp; Behavior Group</td>
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<tr>
<td>• Role in altered sensory signaling, esp. Central (Vestibular) sensing, in altering genome</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Musculoskeletal System</th>
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<tbody>
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<tr>
<th>Growth, Body Size and Composition</th>
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</thead>
<tbody>
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<td>• Sex, Health</td>
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<table>
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<th>Endocrinology</th>
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<table>
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<tbody>
<tr>
<td>• Energetics, Nutrition, Gastrointestinal</td>
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<table>
<thead>
<tr>
<th>Cardiovascular &amp; Blood (i.e., Oxygen transport)</th>
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<table>
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<tr>
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<table>
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<th>Immune Function</th>
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<table>
<thead>
<tr>
<th>External Stimuli</th>
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<tbody>
<tr>
<td>• Exercise, Centrifugation</td>
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<table>
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<tr>
<th><strong>Commercial – Lead: L. Stodieck</strong></th>
</tr>
</thead>
</table>

Number one application is drug or gene therapy testing (interpretations/contingency with off-target effects), in particular, where disease models would apply to young children (e.g., muscular dystrophy, myopathies)

Use of transgenic models would be likely.

Neonates: Model of severe disuse conditions for children

Dam: Model of pregnancy and delivery during conditions of disuse

Pregnant rats on a 4.5-day mission, returning them to Earth prior to birth. This landmark mission provided proof-of-concept that mammalian pregnancy can proceed in the microgravity of space (Ronca, 2003). After a hiatus of more than a decade, NASA and NIH jointly sponsored two secondary payloads on NIH.R1/STS-66 and NIH.R2/STS-70. Sixteen international and domestic science teams analyzed the behavior, morphology, neurobiology, and physiology of ten pregnant rats and their offspring launched at midgestation for 11 and 9 days, respectively. Following recovery, dams had uncomplicated, successful vaginal deliveries and nursed their young. Notably, NIH.R1 and NIH.R2 were model cooperative space biology efforts that fostered novel cross-disciplinary interactions among scientists from diverse disciplines and led to numerous published reports describing reproductive, neural, vestibular, locomotor, immune, musculoskeletal, and circadian factors in dams and offspring (Ronca, 2003).

In 1996 and 1998, the NIH.R3 (STS-72) and Neurolab (STS-90) carried nursing rat dams and
their litters into space for the first time. Sensorimotor, neural, muscular, vestibular, cardiovascular, and cognitive processes were studied in the offspring (Ronca, 2003; Buckey and Homick, 2003). These missions also yielded important “lessons learned” (Ronca, 2003; Maese and Ostrach, 2002). The maternal-offspring system in mammals is exquisitely sensitive to changes in gravity, particularly during the early postnatal period when infants are dependent upon maternal care for their survival. Flight conditions and hardware, including caging, food delivery, and waste removal, therefore exert extraordinary influences on the animals’ health and development. Significant mortality and feeding difficulties were observed in young infant rats flown on NIH.R3 and Neurolab missions, requiring intervention from the astronauts (Maese and Ostrach, 2002). Similarly, quail fledglings hatched on the Russian Space Station Mir were unable to regulate their body position in space, requiring cosmonaut assistance to accomplish feeding (Jones, 1992). These experiments identified a clear need for specialized habitats for flying young postnatal animals in the weightless space environment. The highlights of these efforts are:

Pregnancy and Development Can Proceed in Microgravity

Animal Enclosure Modules (AEMs) onboard the shuttle adequately supported late-pregnant rats. Pregnant rats that experienced spaceflight and were subsequently returned to Earth within 48-72 hrs of normal birth underwent delivery at the expected time. The duration of the birth process was similar in spaceflight-exposed and ground control rats although spaceflight dams exhibited two times more labor contractions.

Suckling Can Occur in Weightlessness

Pups suckled on anesthetized dams during 25 seconds of weightlessness during parabolic flight on NASA’s KC-135 airplane and stayed on the nipple during parabolic maneuvers, obtaining milk. Dams were injected with oxytocin that successfully caused milk letdowns during the parabolic flight. Pups showed milk-letdown reflexes, stretching and extending their hindlimbs, but they remained attached – with their bodies “out in space” around the mother’s ventrum during periods of microgravity and hypergravity that occur in the parabolic flight. Suckling also was demonstrated during the Neurolab mission, STS-90, in 1998.

Younger Neonates Flown on Shuttle Were Adversely Affected by Spaceflight As Compared to Older Pups

STS-72 (NIH.R3) 5-day-olds (housed in the Nursing Facility AEM-NF) and STS-90 (Neurolab) 8-day-olds held in the Research Animal Holding Facility (RAHF) within the Shuttle/Spacelab had high mortality rates and low body weights. Habitat design played a crucial role in neonatal survival in microgravity, particularly in the youngest neonates (Maese and Ostrach, 2002; Ronca, 2003).

Reproductive Biology in Space – J. Tash

Cosmos 1129—Attempted rat breeding during flight: After landing no evidence of pregnancies or fetuses.

Males mated within 5 days of landing with new females produced offspring, but pups were developmentally delayed: (evidence of epigenetic influence on males?). Males mated 2 months post-flight produced normal pregnancies and offspring (no developmental anomalies support epigenetic effect on sperm). No data are available from follow-on assessments of females. There was no video monitoring during this flight. Therefore, it was not possible to distinguish failed fertilization from failure to mate. Ground controls may have also failed to become pregnant (Keefe, 1985). There was no assessment of hardware design to ascertain whether mating could be accomplished in the habitat/caging provided.

Results from STS-131, -133, and -135 (12-15 day missions)

The three experiments used female C57BL/6 or BalbC mice. Microgravity negatively impacted ovarian histology in mice as evidenced by lower numbers of corpora lutea, and unhealthy oocytes. Estrogen receptor levels were lower in flight mice, while gene expression of the HSPH-1 stress marker was down regulated. Oocyte maturation and production were blocked or terminated after 12-15 days (3 estrous cycles) of spaceflight (post-flight recovery has not been ascertained). These findings raise the possibility that COSMOS 1129 rats had no eggs to fertilize and/or refused to mate in absence of estrogen-dependent mating
behavior, though the sensitivity of female mouse and rat reproductive factors to spaceflight is unknown.

Male Reproductive Status in Hindlimb Unloaded Rats

Since its inception at the NASA Ames Research Center in the mid-1970s, many laboratories around the world have used the rat hindlimb unloading (HLU) model to mimic the effects of weightlessness and to study various aspects of musculoskeletal loading (Morey-Holton et al., 2005). In this model, the hindlimbs of rodents are elevated to produce a 30 degrees head-down tilt resulting in a cephalad fluid shift and to avoid weight-bearing by the hindquarters. Long-term HLU inhibits spermatogenesis in adult male rats in the absence of cryptorchidism, changes in testicular function due to hyperthermic testes (Tash et al., 2002). All HLU animals were sterile in 2 mating attempts whereas controls were 100% fertile. Chronic testicular hyperthermia was observed with temperature elevated above normal by 2.2ºC, P<.00001). Other findings included: (1) Invasion of inflammatory cells (≥3 weeks), (2) Catastrophic apoptosis in the testes, (3) These factors cause aspermatogenic dysfunction.

Behavioral Biology – J. Alberts

Life Styles of Rats and Mice

Less information is known for mice – much more is known for rats.

Commensal Habits of Norway rats (Rattus norvegicus) and House Mice (Mus musculus)

Rats live in colonies with multiple males and females. They are characterized by a promiscuous mating system – males mate with multiple females; females mate with multiple males. Mice live in demes with a single territorial male maintaining a range that encompasses homes of multiple females with which it mates. Females may mate with multiple, territorial males. Both species rely on maternal behavior only for rearing young (compared to a few rodents, such as gerbils and some voles that exhibit biparental care). Mice are known for both maternal aggression, with which lactating dams vigorously defend nest and young against males, and for their willingness to engage in “communal rearing” of young, in which they brood and nurse each others’ offspring along with their own.

Colonial life of rats is associated with high levels of tolerance in close proximity (a “contact species”), seen in both females and males, although males are larger and tend to be more aggressive. Social organization of mice is associated with lower levels of tolerance among unrelated, unfamiliar adult males. Amicable contact behaviors are frequently seen among female mice but are not well understood.

Courtship and Copulation in Rats

Females exhibit an estrous cycle, 4–5 days continuously. Some seasonality is suspected but cycling occurs throughout the year in the lab. Females are sexually “receptive” during the estrus phase of the cycle, but they are more accurately defined as proceptive—because the estrus female actively solicits the male’s sexual attention. Female proceptive behavior includes: Moving into vicinity of male, making available their arousing, and estrus odors, which attract male approach behavior and sniffing. The female then “darts” away and stops, the male approaches again, the female darts and “ear wiggles.” The “dance” continues and escalates until copulation occurs.

During copulation: The male mounts and the female exhibits lordosis, making the pudenda more accessible and intromission more likely. Males may intromit and thrust, and multiple intromissions and thrusts culminate in ejaculation. Number and timing of intromissions contributes to fertile matings and reproductive success. Females control timing by retreating and regulating access and modulating their bouts of solicitation. Females show a host of mechanisms and suspected adaptations related to copulation and reproductive success including: (1) Odor-induced pregnancy blockades, (2) Odor-induced resorption, (3) Estrus synchrony (questionable by recent analyses), and (4) Delayed implantation.

Other Considerations:

- Biomechanics of copulatory sequences are worth examining in relation to habitat configuration and implications for performance in microgravity
- Some strain differences exist in the form of copulatory moves, such as mounts, clasps, dismounts, and grooming sequences (known especially for mice)
Ronca, et al. -- ISS Rodent Mark III Habitat Workshop

- Surprising lack of information on interruptions of estrous cycle by general “stress” factors; this would be worth investigating
- Some data exist on habitat configuration (at 1g) and patterned mating in rats, but comparable information has not been collected for mice

Neurolab & Developmental Biology – D. Riley

The STS-90 Neurolab Mammalian Development Team consisted of six principal investigators. Collectively, the experiments had 12 cross-fostered litters of PN8 and PN14 Sprague Dawley rats in each of the flight, asynchronous ground control, and vivarium control conditions. The initial assignment of litters as primary for specific investigators was discarded due to the high in-flight mortality of the PN8 pups, causes unknown, that necessitated reapportioning to maximize science return.

Results and Lessons Learned from Neurolab

The effects of spaceflight on development ranged from unimpaired to permanently altered. Interpretation of these results as to whether gravity is required for normal development must be tempered by the facts that all of the pregnant flight animals experienced at least one week of gravity just prior to launch, and the flight duration of 16-day gravity deprivation was likely too brief to have a major impact on some systems.

Effects of spaceflight on development and growth—the space-flown rodents were pre-exposed to gravity, and the exposures to the spaceflight environment were too short to complete development, growth, maturation, and aging such that deficiencies would manifest. Some findings indicate existence of critical periods requiring gravity environment stimulation for normal (1g) development and growth of systems. Studies of rodents generated in space and never exposed to one-gravity are necessary to elucidate the full impact of the spaceflight environment on development, maturation, and aging (i.e., studies across the lifespan and multiple generations in space). Return of live space-reared organisms is necessary for assessment of exposure to 1g stress to ascertain system weaknesses and repair capacities and the existence of irrevocable structure and function. An on-orbit centrifuge is needed to determine the level of gravity exposure (0-1g) sufficient for 1g comparable development. Science return is enhanced by forming integrated-discipline teams and by judicious sharing of litters. On-orbit animal microsurgery, complex tissue processing, and stable storage of specimens is feasible. Sample return is required for full analysis requiring techniques unavailable in space. Rodent habitat redesign is essential to maintain the neonate and dam interactions that facilitate adequate nutrition, sleep, body temperature control, and other factors of viability provided in a nest environment on Earth (Ronca, 2003).

Alternate Platforms: Chronic Acceleration and Hindlimb Unloading (HLU) – C. Fuller

Chronic Acceleration Model

Life on Earth evolved with a single environmental constant, gravity (1g). Chronic acceleration or hypergravity is the resultant of 1g Earth gravity combined with centrifugal force. The UC Davis Chronic Acceleration Research Unit (CARU) provides an experimental facility for exposing animals to short or long duration hypergravity.

Hypergravity has been used to establish the “Principle of Continuity” (Wade, 2005), the idea that gravitational fields are continuous above and below the gravitational field on Earth, and that biological responses to changes across the spectrum of gravity exhibit a similar continuity. On Earth (1g), fractional increments in g-load exceeding 1g can be continuously applied for extended periods, and dose-response relationship established. While the principle of continuity has not been rigorously tested and validated across the gravity continuum, there is a sizeable corpus of data suggesting that the principle is valid across multiple systems. Reproduction and development, size/growth, energy and metabolism, musculoskeletal, cardiovascular, immune, and CNS sensory/vestibular responses are among those that have been studied in space.

The lack of definitive data on exposure to centrifugation in space for rats and mice makes the extrapolation of the continuum to levels below Earth-gravity problematic. However, in systems where responses are detected for both spaceflight and acceleration by centrifugation, a gravitational continuum is present, supporting the Principle of Continuity or a systematic dose-response
relationship between gravity load and the magnitude of physiological response. Accordingly, the use of hypergravity holds significant potential for predicting responses to spaceflight.

_Hindlimb Unloading Model_  
This model simulates certain features of microgravity exposure by elevating the hindquarters 30° to produce head-down tilt. Support structures are unloaded (bone, muscle), and cephalic fluid shifts are induced. Thus HLU mimics spaceflight effects for certain key systems. Limitations of the technique include immobility of the animals, no limitations or alterations of CNS sensory input such as that which would typically occur in spaceflight.

_Breeding_  
Will females hormonally cycle in the novel space environment? If females cycle, will they solicit and will males respond? In microgravity, can pairs manage mechanics of repeated intromissions needed for ejaculation?

_Mitigation of Breeding Risks_  
Use proven breeders. Cage design should incorporate the biomechanics of mating. Test specific strains of each species in a flight-like habitat. Include some relevant environmental stressors (noise, thermal spikes, lighting) and evaluate breeding success.

_Birthing_  
Detrimental stress effects on the gestating offspring seem likely for both rats and mice. Disrupted onset and initiation of maternal behavior patterns is a major risk (unaddressed by Neurolab experiences), although maternal behavior was intact in late-pregnant dams exposed to spaceflight, then returned to Earth just prior to birth. If there is an absence of nesting material, then we must understand implications of depriving dam of nest building experience as part of maternal behavior and, importantly, the thermal consequences of no nest insulation!

_Mitigation of Birthing Risks_  
It is vital to collect data on thermal requirements of both rats and mice in absence of nesting material. Collect quantitative and qualitative data on mouse parturition in selected strains.

_Nursing/Weaning_  
For the dam—the energetic demands of lactation are considerable, so there must be an adequate balance of energy intake and output (including that for milk production). Adequate diet (enriched in most labs) is essential, as well as feeders capable of delivering more food than is reasonable to expect. For the first 12-14 days postpartum, the dam approaches a unified group of pups. This is important because all nipples acquire milk at once per letdown and thus all pups should be attached to get their fair share. Neurolab revealed the difficulties of maintaining litter integrity. For pups—ready access to dam from Day 14 to 28, as well as appropriate presentation of food for weaning.

_Mitigation of Nursing/Weaning Risks_  
Collect data on energetics of lactation cycle and pup growth under flight-like thermal conditions. Anticipate food presentation and quantities appropriate for dams and for weanlings.

_Two additional presentations were made. Mike Hines presented the current rodent habitat designs for transport to the ISS on the Space X Dragon, the Animal Enclosure Module –Transfer (AEM-T) and housing on the ISS (AEM-X). Cecelia Wigley presented ISS Mission Characteristics including launch, concept of operations, and Recovery. These presentations can be viewed at URL (http://spacebiosciences.arc.nasa.gov/media/Mark3_v1.14_signed_rotated.pdf)  

_Roadmap to Multiple Generations in Space—A. Ronca_  
The workshop concluded with a plan for achieving lifespan and multigenerational rodent studies on the ISS. Spaceflight research offers unique insights into the role(s) of gravitational forces omnipresent on Earth, but absent in orbital flight. These forces may actively shape genomes in ways that are heritable. The 2011 NRC Report determined that studies of structural and functional changes induced by exposure to space during development and the transmission across
Table 3. Concerns, Risks, and Knowledge Gaps from the Workshop Discussion on Breeding, Birthing, and Postnatal Development in Space

<table>
<thead>
<tr>
<th>Subcategory</th>
<th>Basic Science (S) / Hardware (H) / Enabling Science (ES)</th>
<th>Concerns, Risks, &amp; Knowledge Gaps</th>
<th>Risk Mitigation Approaches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-cutting issues</td>
<td>S</td>
<td>What are the changes in endocrine status, including HPG axis and prolactin during spaceflight?</td>
<td>Artificial Insemination</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Are there fertilization issues during spaceflight?</td>
<td>Localized dosimetry at or near (or that closely resembles the shielding characteristics of) the habitat and any exposure from DXA machine.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Does the elevated radiation level affect reproduction or fetal/neonatal development?</td>
<td>Video and monitoring of key parameters</td>
</tr>
<tr>
<td></td>
<td>H, ES</td>
<td>Information is needed to enable the understanding of behavior and of experiment failures.</td>
<td>Define and measure indices of survivability and thrivability</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>Does the environment (i.e., air circulation, noise) within the habitat impede acoustic and olfactory communication between neonates and dam?</td>
<td>Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>Required crew access to animals should inform cage design and procedures (science, safety, etc), see SRED.</td>
<td>Pair feeding. Measure food and water intake.</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>Cage design will play an important role in promoting or inhibiting success of all categories of reproduction and development.</td>
<td>Generate data from a Mark I experiment.</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>Does the current diet present any nutritional deficiencies impeding aspects of reproduction?</td>
<td>Continue using rats to plan validation flight study and to test new hypothesis. Replicate some of the earlier rat-studies with mice in a stepwise fashion.</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>What are the enrichment requirements for Breeders?</td>
<td>Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>What is the acclimation period before harem are ready to mate?</td>
<td>Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>How long have prior foodbar tests/studies been performed? Are there any foodbar inadequacy or stability issues with long-term use of the foodbar?</td>
<td>Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>Most of the knowledge is from studies using rats. Issues can emerge from extrapolation to mice.</td>
<td>Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control</td>
</tr>
</tbody>
</table>

| Mating Behavior              | S                                                        | Is copulation affected by space flight?                                                       | Selection of proven breeders or breeding pairs                                          |
|                              | S                                                        | Is estrous cycling altered by space flight?                                                   | Selection of proven breeders or breeding pairs                                          |
|                              | H, ES                                                    | Are pre-mating behaviors and courtship affected by space flight or the habitat design?        | Selection of proven breeders or breeding pairs                                          |

| Lactation                    | S                                                        | What are the changes in endocrine status during spaceflight, including HPG axis and prolactin? | Observing through skin; milk bands (acquiring milk); tooth eruption. Gauging milk let-downs through video |
|                              | S                                                        | Is the quality and quantity of milk altered during space flight?                             | Pre- vs. during-flight comparison                                                        |
|                              | S                                                        | Do changes in immune function (antibody status in milk; absorption) affect lactation?       | Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control |
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<tr>
<td>H, S</td>
<td>G, R</td>
<td>Are nursing behaviors of the dam and pup, including suckling and retrieving, altered during space flight?</td>
<td>Consider cage design to promote these behaviors. Use video to analyze behaviors. Also, apply a pre-flight candidate selection filter of the dam (define whether she is a gatherer, or not, and define the quality of her milk).</td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>C, G</td>
<td>Is the foodbar adequate for lactation?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Fertilization</td>
<td>S</td>
<td>G, R</td>
<td>Are gonad and gametogenesis and their functions compromised during space flight?</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Is the endocrine status, including HPG axis, altered by spaceflight environment?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Are post-gonad gamete formation, function, and maturation compromised?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertilization</td>
<td>S, ES</td>
<td>G</td>
<td>Does the current diet present any nutritional deficiencies impeding aspects of fertilization?</td>
<td>Pair feeding. Measure food and water intake.</td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Are sperm-egg signaling and interactions compromised?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Is signaling in the fertilized egg compromised by mechanisms including altered gene regulation and DNA damage?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantation</td>
<td>S</td>
<td>G, R</td>
<td>Does mating trigger proper signaling to prepare uterine epithelium for implantation?</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Are adhesion and implantation gravity dependent?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Are uterine epithelial health and stem cells compromised?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placentation</td>
<td>S</td>
<td>G, R</td>
<td>Does decreased connexin-43 levels affect placentation during spaceflight?</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>During spaceflight, does placentation depend on changes in vascular tone?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>Are the signals for placental formation intact?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>During spaceflight, does placentation depend on changes in the immune system?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>G, R</td>
<td>What are the statuses of prolactins and other pro-placentation endocrine signals during spaceflight?</td>
<td></td>
<td></td>
</tr>
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<th>Risk Mitigation Approaches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organogenesis</td>
<td>S</td>
<td>G, R</td>
<td>Are organ formation, maturation, and function compromised during spaceflight?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>G, R</td>
<td>Are the endocrine-driven fetal-development phases that define the sex of offspring altered during spaceflight?</td>
<td></td>
</tr>
<tr>
<td>Birth (also see cross-cutting)</td>
<td>S</td>
<td>R</td>
<td>Will reduced strength in abdominal musculature affect birth?</td>
<td>Caesarean section</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>C, R</td>
<td>Will altered uterine contraction strength affect birth?</td>
<td>Cage design - wall surface texture and grip; dimensions of birthing space</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>G, R</td>
<td>Does the likelihood of a successful birth depend on genetic strain of rat?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H, S</td>
<td>G, R</td>
<td>Is the maternal care pattern at birth intact?</td>
<td>1 hour of pup cooling - temperature regulation; Video and temperature monitoring; space for the young</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>C</td>
<td>With regards to testosterone formation, the temperature exposure of male pups should be closely monitored and potentially regulated.</td>
<td>Include capability for thermography of the pups within the habitat.</td>
</tr>
<tr>
<td>Perinatal Development</td>
<td>H, S</td>
<td>G, R</td>
<td>Are the pups receiving milk in similar manner as ground controls?</td>
<td>Observing through skin: milk bands (acquiring milk); tooth eruption. Gauging milk-let downs through video</td>
</tr>
<tr>
<td>(PND 0-8)</td>
<td>H, ES</td>
<td>G</td>
<td>Is the amount or quality of sleep that the neonates receive affected by spaceflight or cage design?</td>
<td>Video</td>
</tr>
<tr>
<td></td>
<td>H, ES</td>
<td>C</td>
<td>In case of cannibalism, how to quantify the number of pups that were initially born?</td>
<td>Video. Count # that are born. Higher frequency of monitoring around key milestones.</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>C</td>
<td>How will individual pups be identified for further monitoring?</td>
<td>Ink in footpad or tail tattoo/marking, toe-clipping. Crew access. ANG; coat color.</td>
</tr>
<tr>
<td></td>
<td>ES</td>
<td>C</td>
<td>Body mass shall be recorded 2x/week.</td>
<td>Repeated measurement of body mass is important during the first few weeks post-natal to assess growth.</td>
</tr>
<tr>
<td>Infant Development</td>
<td>S</td>
<td>G</td>
<td>Do changes in the development of ovaries and testes occur during spaceflight?</td>
<td></td>
</tr>
<tr>
<td>(PND 8-14)</td>
<td>H, S</td>
<td>G, C</td>
<td>Is the amount or quality of sleep that the neonates receive affected by spaceflight or cage design?</td>
<td>Video</td>
</tr>
<tr>
<td></td>
<td>H, ES</td>
<td>C</td>
<td>Huddle formation needs to be considered during cage design, as it is an integral aspect of neonatal development</td>
<td>Cage design (artificial nest)</td>
</tr>
<tr>
<td></td>
<td>H, ES</td>
<td>G</td>
<td>Does the relative humidity need to be regulated within a specific range for neonates?</td>
<td></td>
</tr>
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<tbody>
<tr>
<td>H, ES</td>
<td>G</td>
<td>Are there thermal-regulation requirement differences between pups and mother?</td>
<td>Cage design (compartments with unique thermal regulation). Thermography capability needed.</td>
<td></td>
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<tr>
<td>Pre-Weaning</td>
<td>S</td>
<td>G</td>
<td>Do changes in the development of ovaries and testes occur during spaceflight?</td>
<td></td>
</tr>
<tr>
<td>(PND 15-21)</td>
<td>S</td>
<td>C</td>
<td>Milestone: Independent ingestion should occur around PND15 (Neurolab).</td>
<td></td>
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<tr>
<td>Adolescence</td>
<td>ES</td>
<td>C, R</td>
<td>Consider the risk of impregnation of siblings at sexual maturity when establishing age to separate the litter.</td>
<td>Male-female separation Mice: approx. 21-35 days Rats: approx. 45-80 days</td>
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generations of such effects are a major priority for Reproduction and Developmental Biology research in space promising fundamental new knowledge about how genetic and epigenetic factors interact with the environment to shape gravity-dependent processes, other changes induced by the space environment, and their penetrating influence(s) across generations.

The Roadmap to Multiple Generations in Space calls for a staged approach with defined milestones involving ground-based and spaceflight efforts to address habitat development and enabling science gaps in the specific areas of: (1) Breeding, (2) Birth through Weaning, then (3) Multiple Generations (Figure 2). Ideally, singular flights will verify that breeding, birthing, and weaning occur successfully before multiple generations are attempted. At each step along the roadmap, cross-disciplinary translational science will be possible and encouraged.

Figure 2. Roadmap to Multiple Generations (Rat) in Microgravity. AEM T (Animal Enclosure Module–Transfer); AEM X (Animal Enclosure Module–ISS Housing).

Figure 3. Multigenerational Timetable and Earth/Space Exposures. If F0 (parents) are bred on ISS, the first totally space-adapted organisms (and their germ-cell lines) will be the F2 generation.
CONCLUSIONS

The Mark III Rodent Habitat Workshop assembled a diverse range of science, engineering, and program experts to identify necessary requirements and specifications for a flight habitat to increase the probability of successful mating, birth, and development of rodents in space. Detailed discussions of existing knowledge, research gaps, risks, and risk mitigation at key reproductive and developmental phases, and comparison of rats versus mice enabled the group to reach consensus on the development of top-level science requirements and an expansion of the ISS Rodent Research Science Requirements Envelope Document (SRED) (URL: Pending) to include detailed requirements for Reproductive and Developmental Biology.

The recommendations set forth in the 2011 National Academy of Sciences Report, Recapturing a Future for Space Exploration: Life and Physical Sciences Research for a New Era, call for reproductive and developmental biology research within and across generations. The workshop participants uniformly agreed that, prior to embarking on multigenerational studies, individual “milestones” should be met for distinctive reproductive and developmental phases to ensure success across these life stages. As depicted in the “Roadmap to Multigenerational Studies” (Figure 2) an intermediary achievement will be a full mammalian life cycle in space, involving successful mating, pregnancy, birth, lactation, suckling, weaning, and postnatal development to adulthood. Work needs to be accomplished, starting now, in each of these areas, especially to close high priority knowledge gaps (Table 3). In addition to ground-based efforts, important project milestones could be achieved through a sequence of three validation flights that will also address the specific goals of: (1) Breeding, (2) Birth through Weaning, and (3) Multiple Generations. Multigenerational success is a repeating cycle of necessary milestones. The capstone of these efforts will be the first breeding, birth, and development of purely space-grown mammals opening the door to unique opportunities to investigate the role and influence of gravity on a complex organism, the rodent.

ACKNOWLEDGEMENTS

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REFERENCES


Index of Authors

<table>
<thead>
<tr>
<th>Name</th>
<th>Page</th>
<th>Name</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alwood, Joshua S.</td>
<td>107</td>
<td>Lloyd, Shane A.</td>
<td>2</td>
</tr>
<tr>
<td>Bateman, Ted A.</td>
<td>2</td>
<td>Massa, Gioia</td>
<td>95</td>
</tr>
<tr>
<td>Benjaminson, Morris A.</td>
<td>51</td>
<td>Morrow, Robert C.</td>
<td>95</td>
</tr>
<tr>
<td>Caro, Janicce L. Chevez-Barrios, Patricia</td>
<td>29</td>
<td>Nier, Heath A.</td>
<td>20</td>
</tr>
<tr>
<td>Dunlap, Alexander W.</td>
<td>2</td>
<td>Ono, Kumiko</td>
<td>47</td>
</tr>
<tr>
<td>Ferguson, Virginia S.</td>
<td>2</td>
<td>Prospero Ponce, Claudia Maria</td>
<td>29</td>
</tr>
<tr>
<td>Forsman, Allan D.</td>
<td>20</td>
<td>Ronca, April E.</td>
<td>107</td>
</tr>
<tr>
<td>Gilchriest, James A.</td>
<td>51</td>
<td>Sanzari, Jenine K.</td>
<td>79</td>
</tr>
<tr>
<td>Gkigkitzis, Ioannis</td>
<td>59</td>
<td>Simske, Steven J.</td>
<td>2</td>
</tr>
<tr>
<td>Globus, Ruth K.</td>
<td>107</td>
<td>Souza, Kenneth A.</td>
<td>107</td>
</tr>
<tr>
<td>Gridley, Daila S.</td>
<td>79</td>
<td>Stutte, Gary W.</td>
<td>95</td>
</tr>
<tr>
<td>Haranas, Ioannis</td>
<td>59</td>
<td>Tanigaki, Y.</td>
<td>47</td>
</tr>
<tr>
<td>Hummerick, Mary E.</td>
<td>95</td>
<td>Theriot, Corey A.</td>
<td>29</td>
</tr>
<tr>
<td>Kennedy, Ann R.</td>
<td>79</td>
<td>Wan, X. Steven</td>
<td>79</td>
</tr>
<tr>
<td>Krigsfeld, Gabriel S.</td>
<td>79</td>
<td>Wheeler, Raymond M.</td>
<td>95</td>
</tr>
<tr>
<td>Kuniyoshi, H.</td>
<td>47</td>
<td>Wroe, Andrew J.</td>
<td>79</td>
</tr>
<tr>
<td>Lehrer, Stanley</td>
<td>51</td>
<td>Zanello, Susana B.</td>
<td>29</td>
</tr>
<tr>
<td>Livingston, Eric W.</td>
<td>2</td>
<td>Zouganelis, George D.</td>
<td>59</td>
</tr>
</tbody>
</table>