Gravity as a Continuum

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Topical Sentence Gravitational acceleration - from microgravity to hypergravity - is a singular independent variable worthy of study in space-based and ground-based experiments across organisms to better understand gravity's role in life's processes.

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Organisms have evolved in Earth's static gravitational environment. It is not surprising that exposure to altered gravitational environments can extensively alter the regulation of physiology and behavior. Indeed, numerous ground and flight studies have shown effects of altered gravity on a myriad of systems, including nervous/ocular, endocrine, muscle, bone, cardiovascular, metabolism, circadian, sleep, immune, mammary metabolism, reproductive, etc, reviewed by Wade 2005 and recently by Afshinnekoo et al, 2020. Effects also differentially affect life stages, ranging from development through aging (see Ronca, et al's *Multi-Generational* white paper). Past studies cover the translational spectrum from basic biology to countermeasure development.

Hypergravity via centrifugation 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, ocular, and CNS sensory/vestibular responses are among those that have been studied in space. More data are needed to better understand exposure to acceleration using ground-based testing (e.g. disuse or centrifugation) and spaceflight using animal models described below. 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. Thus, hypergravity models hold potential for predicting responses to spaceflight.

In this paper, we **strongly recommend** NASA study Gravity as a Continuum (GAAC) by:

- Utilizing invertebrate and vertebrate model organisms to focus on the gravitational effects on physiological systems, needed by exploration-class missions to the Moon and Mars. Such research may contribute to improve understanding of human-relevant physiology and medicine on Earth.
- Pursuing dose-responses and mechanisms of adaptation to altered gravity.
- Considering artificial gravity as a cross-cutting countermeasure.
- Continuing to partner with international entities to realize GAAC studies.

Dose responses and mechanisms Studies spanning the gravity continuum are needed to establish dose-response relationships and to identify shared mechanistic responses. Important answers that should be pursued include the potential role of oxidative stress and inflammatory responses in adaptation to altered gravity and potential mitochondrial dysfunction associated with spaceflight (da Silveria et al, 2020) linked with phenotypic outcomes, e.g., leaky blood barrier permeability within the brain (Dubayle et al, 2020) (Mao et al, FASEB J, 2020) and retinal systems (Mao et al, 2019).

As an example, the series of studies on the effects of altered gravity on mammary metabolism by Plaut et al, (1999, 2003) identified the influence of gravity. Pregnant animals were exposed to microgravity (0G), control conditions at 1G, and centrifugation at three gravity levels between 1.5G to 2G for eleven days during gestation. On the twentieth day of gestation, glucose oxidation into carbon dioxide and incorporation into lipids was measured in mammary glands, with a strong negative correlation between metabolic rate and the level of gravity: 99% of the variance in glucose incorporation into lipids or oxidation could be accounted for by differences in gravity load. These

findings demonstrated a clear alteration in metabolism across the gravity continuum, and the utility of using hypergravity as a means of predicting responses to spaceflight.

Additionally, the central nervous system (CNS) integrates information from somatic and neural sensory elements that transduce changes in both gravitational magnitude and the orientation of an organism with respect to gravity, reviewed recently by Clement et al, 2020. In addition, following integration, the CNS initiates appropriate physiological and behavioral changes to re-establish homeostasis. Briefly, these changes include: cellular function, CNS development, neuronal metabolism, neurotransmitter levels, vestibular reflexes, and the regulation (e.g., altered CNS efferent outflow) of peripheral physiological systems and behaviors. To this end, much of what we know about CNS changes in altered gravity reflects the use of animal models, including invertebrates, non-mammalian vertebrates, rodents and non-human primates. Although humans are common subjects for neuroscience research during spaceflight, much of the foregoing knowledge could not have been ethically or otherwise satisfactorily collected from human subjects. More recent studies have begun to elucidate the precise somatic and neural sensory elements, e.g., the circadian timing system, that transduce the gravitational environment to affect CNS regulation.

One specific CNS regulatory system, the circadian timing system (CTS), has been of particular interest to the Fuller laboratory. The CTS is responsible for the generation and internal temporal coordination of daily rhythms and for synchronizing these rhythms with the external 24-hour environment, and its regulation is influenced by spaceflight. Individuals with CTS dysfunction often suffer from sleep-wake disturbances, mental health and affect disorders, neuroendocrine dysregulation, and severely compromised long-term health. Recently, Fuller's laboratory elucidated a significant and unique role of the vestibular system in mediating the CTS responses to changes in gravity (Fuller & Fuller, 2006). This novel observation has led to a paradigm shift in our thinking; that is, changes in vestibular loading will likely be a critical component of an appropriate countermeasure for astronauts suffering CTS dysfunction. Furthermore, an influence of the vestibular system on either CTS entrainment or photic responsiveness will have important implications not only for individuals in altered gravity environments, but also for earth-bound individuals with circadian disorders and potentially, vestibular disorders. By understanding the physiological and molecular mechanisms by which the vestibular and circadian systems interact, therapeutic and diagnostic strategies may be developed for astronauts, who are known to suffer from circadian and sleep-wake disorders and for earth-bound individuals suffering from circadian, vestibular, sleep-wake or aging-related disorders. Although not detailed here, we have also identified vestibular signaling of gravitational information as a critical element in metabolic (Fuller, et al. 2002), muscle and immune responses.

Animal Models Animal studies across phyla are critical adjuncts to human studies for the following principal reasons. First, tests with animals can include invasive telemetry, hazardous procedures, and postmortem tissue analysis to understand basic underlying mechanisms and to help define countermeasure prescriptions. Second, animal tests will reduce the total numbers of human subjects needed, and thereby make schedule and cost targets achievable. Animal tests cannot fully replace human tests and campaigns should strive for validation in humans. Third, the large sample size possible using animals to test altered gravity regimens yield results with less scatter (lower error), and thus improve the basis for drawing strong conclusions regarding success or failure of the test conditions. Modeling gravitational effects based on a well-defined set of animal responses allows comparisons to existent mechanical formalisms (e.g., Whalen, Carter, Steele, 1988 for bone density) and further extrapolation to humans.

Non-Human Primates The rhesus monkey provides a biomedical model with close phylogenetic ties to humans. In addition, non-human primates are larger species and enhance the scaling principle in acceleration physiology, which in this context refers to organisms with larger masses demonstrating gravitational sensitivity that is quantitatively more similar to humans than smaller species. Rhesus monkeys have served as subjects in spaceflight experiments, most notably the Cosmos/Bion series of Russian Bioflights. They have been utilized in the study of the responses of numerous physiological systems to alterations in the gravitational environment. Rhesus monkeys have been the subjects of studies on the effects of exposure to microgravity on thermoregulation, immune responses, musculoskeletal system, cardiovascular system, fluid balance, sleep, circadian timing, metabolism, and neurovestibular/neurosensory and psychomotor responses. In ground-based studies, rhesus have served as subjects in experiments utilizing the microgravity models head down tilt (bedrest) and dry immersion as well as gravitational experiments produced via centrifugation, both chronic and intermittent. The systems examined in many of these studies have paralleled those examined during spaceflight. The rhesus monkey confers many advantages as a research subject in the field of gravitational physiology. First and foremost, the rhesus monkey is the most widely accepted biomedical nonhuman primate model for the human. Secondly, the rhesus has a bipedal upright posture, and thus experiences the ambient force environment along the same body axes as the human. Third, the reproductive cycling of the female rhesus is menstrual, similar to humans, and in contrast with virtually all other biomedical models. Fourth, the cognitive abilities of the rhesus monkey allow the use of psychomotor testing to discern the effects of altered gravity on neurovestibular physiology, performance and behavior. Finally, the larger size of the rhesus also allows for collection of larger tissue samples and provides the ability for simultaneous measurement of multiple physiological and behavioral factors.

Rodents Classic mammalian mouse and rat models offers a number of advantages as a model systems for gravitational physiology countermeasure development. Due to small body size rodents are especially well suited to the initial exploratory studies where many permutations of G level, rotational rate, and duty cycle will be explored. With modest caging and care requirements, higher numbers of subjects can be accommodated to increase the statistical power of analyses. Rodents, unlike primates, do not require special isolation or quarantine procedures. Rodents readily adjust to centrifugation and since they can also be used in hind limb immobilization and tail suspension studies, they can also serve as models for deconditioning. Rodents are the most commonly used biomedical research model, and thus a great deal is known about their normal physiology, including characteristics of well-established strains. The relative uniformity of specific strains also presents fewer of the confounding factors that are typical of human studies and thus studies are likely to be both easier to interpret and to repeat. Previous centrifugation and suspension studies also provide a baseline against which gravitational physiology protocols can be evaluated. Similarly, rodents can be used in exercise studies of metered activity using running wheels or treadmills. Certainly, rodents provide opportunities for more invasive or terminal procedures that would not be possible with human subjects, e.g., studies involving both acute and chronic implantation, including use of catheters, electrodes and telemetry. When fully implanted, these also provide the means for completely hands-off data collection, including monitoring of blood pressure and flow, ECG and heart rate, as well as temperature and activity. Rodents can also provide some level of repeated samples of fluids such as blood or urine. Post-mortem tissue sampling is easily accomplished, and at considerably less expense than alternates such as non-human primates. The short generation-time and rapid development of

rodents also lend themselves to developmental and aging studies. Further, the time scale of some changes, for example muscle wasting in microgravity or hindlimb unloading is more rapid than in humans, thus shorter and multiple studies could be accomplished in the same time frame using rodents. Rodents are also relatively well studied in microgravity, and share the advantages of other nonhuman spaceflight subjects in not having conflicting schedules and operational duties to confound experimental findings. Thus, rodents have been important in contributing to our understanding of spaceflight changes in musculoskeletal, neurovestibular, immune, developmental, cardiovascular and metabolic physiology. In collaboration with international partners, rats flown on the Russian Bion biosatellite and mice residing on the JAXA centrifuge (Shiba, et al, Sci Rep, 2017), (Mao, et al, 2018) have provided important spaceflight evidence for the efficacy of 1G centrifugation in preventing many of the degenerative changes seen in microgravity. Since rodents have been among the few species studied in both microgravity and hypergravity, they have provided rare evidence for the direct scaling of many physiological changes with G-level, both above and below the terrestrial level. Validation of artificial gravity (AG; see below) countermeasures in spaceflight will almost certainly begin with rodent studies. With no human-rated centrifuge being flown in the foreseeable future, initial flight-studies using AG for full 1G replacement or conditioning to partial G (Moon, Mars gravity) will necessarily use rodents.

Invertebrates Along with rodents, invertebrates such as *Caenorhabditis elegans* (worms) and *Drosophila melanogaster* (flies) have been established as valuable models for understanding the effects of microgravity and hypergravity (Hateley et al., 2016; Ikenaga et al., 1997; Marcu et al., 2011; Ogneva et al., 2016). The strong evolutionary conservation to mammals, together with the availability of suitable flight hardware, the tractability of genetic tools, cost-efficiency, small size, ease of maintenance, and propagation of large population sizes within the limited volume and mass requirements in space, make flies and worm highly suitable models to study gravity-mediated physiological changes. Additionally, flies are capable of reproducing even when exposed to hypergravity as high as 5G for flies (Hateley et al., 2016), while worms can survive upwards of 400,000G (de Souza and Pereira, 2018) making these model organisms amenable for cost-efficient ground-based gravitational studies. Invertebrate studies have demonstrated inverse effects in micro- and hyper-gravity highlighting differential physiological adaptations to gravity (e.g., divergent changes in collagen biosynthesis; Seitzer et al., 1995; humoral stress pathway; Marcu, et al., 2011 and Hosamani et al., 2016). Conversely, identification of signatures along the gravity continuum suggests conserved responses (e.g., oxidative stress and proteolytic responses; Walls et al., 2020 and Hosamani et al., 2016). Such foundational invertebrate studies can provide insights into molecular mechanisms affected due to spaceflight and altered gravity.

Further, the short generation time and high fecundity of flies and worms will be advantageous in performing long-term multi-generational experiments in space, thus aiding in our understanding of the genetic effects of spaceflight across generations. Recent advancements in *D. melanogaster* flight hardware (MVP-platform, Techshot, validated in MVP-Fly01 mission) also allow for the separation of multiple generations in space, and an on-board centrifuge with the capability to simulate different gravity regimes during spaceflight thus enabling on-orbit assessment of the effects of altered gravity (e.g., Lunar, Mars, Earth gravity, and hypergravity) in comparison to microgravity effects. Similarly, proven flight hardware for *C. elegans* allows multigenerational growth in space (CHab, Bioserve), assessment of multigenerational gene expression in flight (ECs, ESA, STaARs and CBEF, JAXA), and onboard centrifuges allowing examination of the effect of altered gravity in-flight (CBEF, JAXA and KUBIK, ESA).

Artificial Gravity Among the considerations for development of an integrated countermeasure (CM) to protect astronauts in space, gravitational loading is potentially of the greatest physiological value since it replaces the most characteristic terrestrial stimulus lost during spaceflight (Clement & Buckley, 2007). Since Tsiolkovsky, artificial gravity (AG) has been proposed as a countermeasure for deconditioning-related changes and for other physiological/behavioral effects of living in space. The effectiveness of AG should be thoroughly experimentally determined. We do not yet understand exactly how AG will be used in space; what is the prescribed duty cycle. The most basic considerations have yet to be evaluated including G level, duration and frequency of exposure, and when to time a G stimulus. The minimum G level required to counteract microgravity-induced changes is not known. At present we know little about the effects of intermediate G levels, for example the 0.38G of Mars or the 0.17G of the Moon. We know from previous lunar missions that intermediate G exposure is survivable and has long-term effects no worse than microgravity exposure, but do not know if intermediate G levels will have beneficial effects or if fields of 1G or greater will be required to prevent long-term deleterious changes. Animal studies are needed to characterize and understand the parameter space.

Platforms Both ground and flight capabilities are required for a thriving space life science program. Flight platforms required by the international community are driven by the questions being investigated. At present, these potentially include the International Space Station including partnering with Japan to use their on-board centrifuge, free-flyer satellites like Bion (partnering with Russia), and, possibly, sub-orbital exposure to microgravity. To take advantage of any of these platforms, the community needs regular and reliable access to them, as well as the necessary support facilities to house subjects, acquire data and return specimens. We recommend continuing these platforms and extending to Gateway and Lunar Outposts and building strong relationships with commercial entities (e.g., SpaceX, Axiom) to carry science to LEO and beyond LEO.

Ground models currently utilize a variety of technologies, including unloading/inactivity via bed rest or hindlimb unloading, and the use of chronic centrifugation. Further, of these ground models it is important to understand that only chronic acceleration provides symmetrical changes in gravitational loading across the organism (i.e., the loading is altered both peripherally and centrally). Amongst the ground support technology that is currently in need of preservation are the large and specialized US animal centrifuge facilities designed for gravitational physiology research. Currently, the only dedicated facilities of this type in the US are at NASA Ames Research Center and the University of California, Davis. Consideration should be given to directly comparing the deconditioning of *animals adapted to hypergravity* (e.g., 2G) once they are returned to normal 1G with animals experiencing spaceflight and/or unloading/inactivity models. Finally, while bedrest and immersion studies are a frequently utilized model for biomedical studies in humans, there are very few facilities currently available to perform chronic gravitational studies in human subjects.

Conclusion We believe it is crucial for life sciences research to continue to allow for full exploration of space and understanding of the effects of space flight conditions - especially gravity - on life. The NAS should continue to advocate for a robust program developing both basic, clinical, and translational research in life sciences in space. This program should encompass a substantial ground-based component, as well as flight studies to allow for a full understanding of the effects of gravity on living systems across tissue systems and to allow for safe exploration of space.

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