

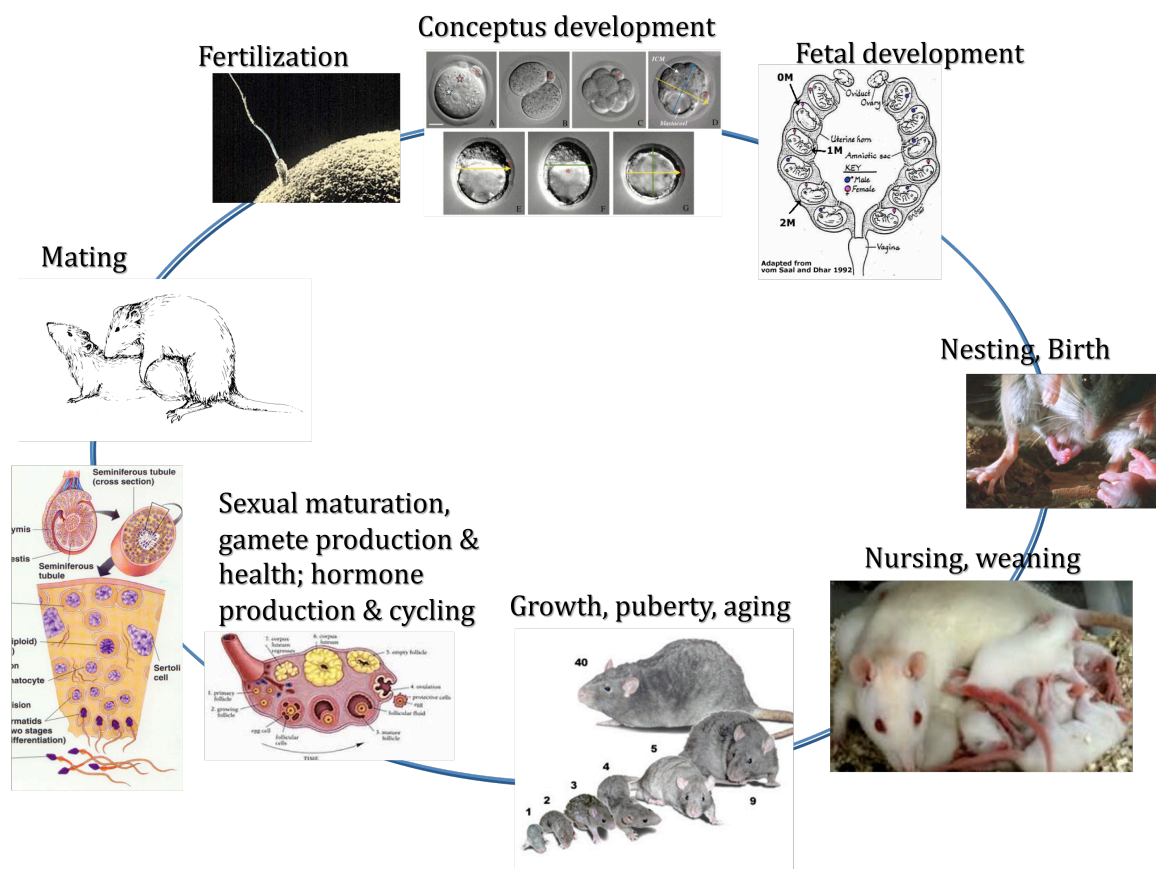
Rodent Mark III Habitat Workshop Final Report

NASA Ames Research Center

March 21-22, 2013

Co-Chairs: Kenneth A. Souza, April E. Ronca

Multigenerational Success is a Repeating Cycle of Necessary Milestones



Workshop was sponsored by the NASA Ames Research Center Fundamental Space Biology Division

Ruth K. Globus, PhD, Rodent Habitat Project Scientist

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I. List of Participants

Jeffrey Alberts, PhD

Indiana University, Bloomington

In absentia

Joshua S. Alwood, PhD

Executive Secretary

Oak Ridge Associated Universities

Ted A. Bateman, PhD

University of North Carolina

Shawn G. Bengtson

McGowan Institute at the University of
Pittsburgh

Allison Brown

LifeSource Biomedical, LLC

Kristin D. Evans, PhD, DVM

University of California, Davis

Charles A. Fuller, PhD

University of California, Davis

Ruth K. Globus, PhD

NASA Lead, NASA ARC

Mike Hines

NASA ARC

Danny A. Riley, PhD

Medical College of Wisconsin

April E. Ronca, PhD

Workshop Co-Chair

Wake Forest School of Medicine

Stephanie Solis, DVM

LifeSource Biomedical, LLC

Kenneth A. Souza

Workshop Co-Chair

Logyx, LLC

Marianne Steele, PhD

Lockheed Martin

Louis S. Stodieck, PhD

BioServe Space Technologies

University of Colorado at Boulder

Joseph S. Tash, PhD

University of Kansas Medical Center

II. Agenda

March 21, 2013

8:00am Opening Remarks, Charter and Introductions - K. Souza
8:20am Historical Perspective and NRC Decadal Survey - A. Ronca
9:00am Reproductive Biology in Space - J. Tash
9:30am Behavioral Biology - J. Alberts* (presented by A. Ronca)
10:15am Neurolab & Developmental Biology - D. Riley
10:45am Alternate Platforms including Chronic Acceleration & HLU - C. Fuller
11:15am Breeding, Birthing, and Postnatal Development in Space: Problems & Concerns/Risks and Risk Mitigation. Discussion Leader – A. Ronca
3:15pm Rodent Habitat – AEM T & X Overview - M. Hines
3:45pm ISS Mission Characteristics: Launch, Concept of Operations, Recovery - C. Wigley
4:15pm Day 1 Summary & Day 2 Plan - J. Alwood & K. Souza

March 22, 2013

8:00am Group questions & discussion (Discussion Leaders: A. Ronca & K. Souza)

- What are the major science success criteria for Mark III habitat?
- Which rodent, mice or rats, poses the lesser risks to meeting mission targets (mating, birthing, nursing, weaning) and yielding high science return?

10:15am Science Requirements Envelope Document (SRED), Rev. E: Discuss SRED questions, modifications, and requirements. Define why a Mark III is needed to accommodate rodent reproduction & development. (Discussion Leaders: R. Globus, K. Souza, & A. Ronca)
2:45pm Representative Reproductive and Developmental Experiments that require a Mark III Habitat: (15 min presentations)
3:30pm Discuss Gaps in Knowledge
4:15pm Roadmap to Multiple Generations (A. Ronca)

*J. Alberts was unable to attend

III. Executive Summary – A. Ronca

Workshop Goals

The major goal of the Rodent Mark III Habitat Workshop, held March 21-22, 2013 at NASA Ames Research Center, was to identify top-level science requirements suitable for defining the envelope for the Rodent Mark III Habitat, an animal habitat that will support the reproduction and development of rodents for research on the International Space Station (ISS). The 2011 National Academy of Sciences Report, *'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 unique space environment among the *highest priority NASA initiatives*. Precedence is given to studies of rats and mice examining the transmission across generations of structural and functional changes induced by exposure to the space environment. This directive encompasses the key reproductive and developmental phases comprising the mammalian life cycle. To attain this goal, spaceflight animal housing and hardware development will need to support multiple endocrine and environmental requirements for maximizing successful outcomes for: (1) mating, (2) conception, (3) pregnancy, (4) embryonic/fetal development, (5) birth, (6) lactation (7) maternal care, and (7) offspring development through sexual maturity. Achieving these individual milestones and their repeating cycle will form the foundation for multigenerational success.

The Rodent Mark III Habitat Workshop assembled a diverse team of experts 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. On the first day, participants received overview presentations reporting relevant science, engineering, and space operations information. They began discussions of concerns, potential risks, and risk mitigations corresponding to each reproductive/developmental stage. On the second workshop day, team leaders presented specific examples of research within the major space bioscience disciplines requiring a Mark III habitat to achieve their objectives. However the lion's share of the day 2 discussions focused on expanding the ISS Rodent Research Science Requirements Envelope Document (SRED) to include detailed requirements for Reproductive and Developmental Biology that will provide the segue to multigenerational research. The Workshop concluded with the presentation of a 'Roadmap to Multigenerational Research' that was fine-tuned by the participants to create a solid plan for multigenerational success.

Conclusions and Recommendations

Relevant flight experiments were suggested spanning important and timely research in Reproductive Biology, Neuroscience and Behavior, Musculoskeletal System, General Physiology and Immunology, and Commercial Research. There was consensus on the importance of emphasizing rodent models with strong translational relevance to human disease, including those affecting infants and children and 'developmental programming' of later life disease. Sexual definition and development, the achievement of reproductive/developmental milestones, identification of critical periods of development, epigenetic alterations, and stem cell production were among the highlighted research areas for space biosciences research utilizing a Mark III Rodent Habitat.

Top-level requirements were identified specific to the mating, birthing, nursing, and maturation of rodents on the ISS, both within and across generations. The participants recommended that studies should initially focus on rats rather than mice given that more is known about rat maternal behavior, reproduction, and development both during spaceflight and on Earth. Concerns were also raised that the quality of mouse maternal care under flight conditions could be reduced because mouse dams are more sensitive to environmental conditions and have more difficulties raising robust litters. It was agreed that proven breeders should be flown. Numerous specific recommendations were discussed and used to establish representative experimental details and the full range of the science envelope. Within-habitat environmental parameters were considered for each reproductive and developmental phase incorporating the guidelines set forth in the 2011 National Research Council Guide for Care and Use of Laboratory Animals, 8th Edition and existing scientific literature. Recommendations for ground-based research needed to close gaps in knowledge, where appropriate requirements cannot be specified, were identified.

Finally, the workshop participants unanimously agreed that, prior to embarking on multigenerational studies, individual project ‘milestones’ should be met to ensure success across key reproductive and developmental life stages. As depicted in the ‘Roadmap to Multigenerational Studies’ (page 37 of this report), 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 knowledge gaps presented on page 23 of this report. In addition to ground-based efforts, important project milestones could be achieved through a sequence of three hardware validation flights that will address enabling science gaps in the specific areas 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 conception, birth, and development of purely space-grown mammals.

IV. Charter and Introduction – K. Souza

Purpose

To prepare top level science requirements for the development of an animal habitat (Rodent Habitat Mark III) that will support the reproduction and development of rodents for research on the ISS.

Approach

A workshop of approximately 20 participants met for 2 days at the Ames Research Center, on March 21-22, 2013. The workshop included experts in animal husbandry (rodents), animal behavior, reproductive and developmental biology, animal physiology, veterinary medicine, flight hardware development, and spaceflight operations. In preparation for the workshop the participants were asked to review the NASA Rodent Research Science Envelope Requirements Document (SRED), Rev. 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 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 ISS AEM-T and AEM-X rodent habitats 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 requirements necessary for habitat development.
5. Identified specific examples of research that require an RH Mark III habitat.
6. Observed rodent flight-hardware: The nursing insert for the Space Shuttle AEM flown on the STS-72 NIH-R3 mission in 1996 and a prototype Animal Development Habitat developed by STAR Enterprises as a Small Business Innovative Research (SBIR) project in 1986.

Workshop products and follow-on activities

A workshop report has been completed and includes:

- A summary of the discussions and recommendations of the workshop participants
- Specific top level science requirements suitable for defining the envelope for the RH Mark III habitat
- Recommendations for ground-based research needed to close gaps in knowledge where appropriate requirements cannot be specified
- Examples of flight experiments that are consistent with the NRC Decadal Survey 2011 recommendations and would require RH Mark III habitats to achieve their objectives.

The workshop report will be used to expand the current ISS Rodent Research SRED (Rev. 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.

V. Workshop Presentation Summaries

Historical Perspective – A. Ronca

Between 1979-1998 six experiments were conducted to investigate reproductive and developmental biology during space flight (Figure 1). All experiments were short-duration space flight between 4.5 days to 18.5 days in duration, covering mating and conception and pre- and post-natal development. All but one experiment used rats, with one experiment using mice. The list of missions:

- 1979 Cosmos 1129 (18.5 days)
- 1983 Cosmos 1514 (4.5 days)
- 1994 NIH.Rodent (R)1 (11 days; STS-66)
- 1996 NIH.R2 (9 days; STS-70)
- 1997 NIH.R3 (9 days; STS-72)
- 1998 Neurolab (16 days; STS-90)

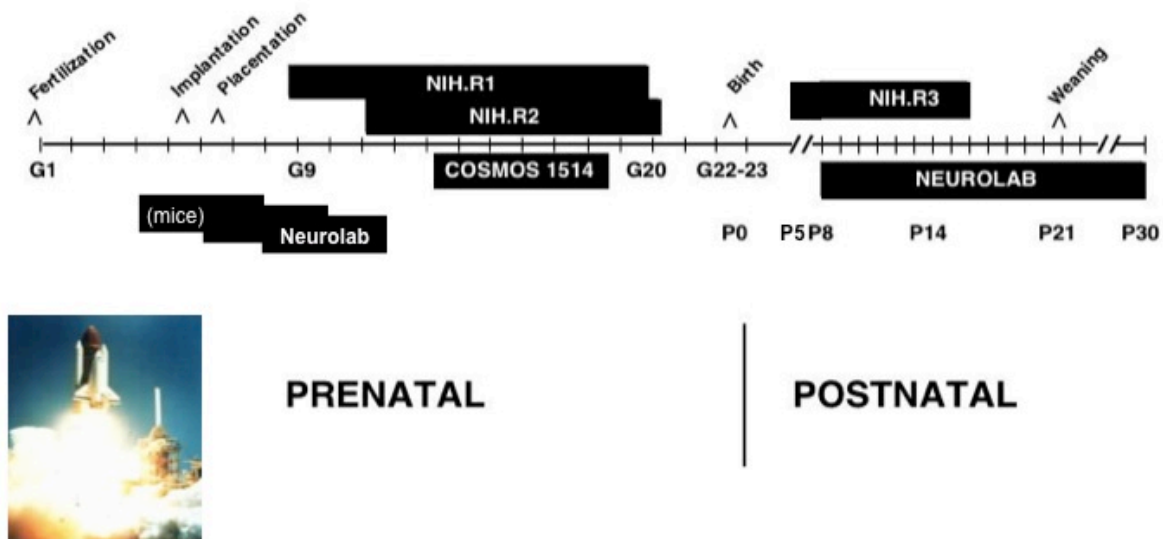


Figure 1 Previous space flight experiments have investigated development both pre- and post-natally.

Mammalian Development Flight Summary

Pregnancy and Development Can Proceed in Microgravity

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

Suckling Can Occur in Weightlessness

- STS-72 (NIH.R3), STS-90 (Neurolab)
- Pups suckled on anesthetized dams during 25 seconds of weightlessness during parabolic flight on NASA's KC-135 airplane.
- Pups stayed on the nipple during parabolic maneuvers and obtained milk.
- Dams were injected with oxytocin that successfully caused milk let-downs 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.

Younger Neonates Flown on Shuttle Were More Affected

- 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 plays a crucial role in neonatal survival in microgravity, particularly in very young neonates.

Decadal Survey – A. Ronca

In 2009, Congress commissioned the National Academy of Sciences to undertake a Decadal Study of the NASA Life and Physical Sciences Research Programs:

“Achieving the goals of the Exploration Initiative will require a greater understanding of life and physical sciences phenomena in microgravity as well as in the partial gravity environments of the Moon and Mars. Therefore the Administrator is directed to enter into an arrangement with the NRC to conduct a “Decadal Survey” of life and physical sciences research in microgravity and partial gravity to establish priorities for research for the 2010-2020 decade.”

Animal and Human Biology Panel

- (1) What is known about the risk and deleterious effects of spaceflight (and ground based analogues) on the structure and function of the musculoskeletal (bone and muscle), sensory-motor, cardiovascular, pulmonary, endocrine, and immune systems, as well as how animals develop in the absence of gravity;
- (2) Effectiveness of the countermeasures currently used to maintain organ system homeostasis in the face of microgravity;
- (3) Gaps in knowledge needed to be addressed in understanding the above topics;
- (4) Research platforms needed to undertake new research initiatives in the next decade;
- (5) Overarching issues that need to be addressed in fostering cutting edge, integrative research in humans and animals spanning multiple physiological systems to generate future countermeasure strategies; and
- (6) Specific high priority research initiatives that are needed to sharpen and advance the science knowledge necessary for progress in the next decade.

Reproduction and Developmental Biology Research Priorities

- Studies should be conducted on transmission across generations of structural and functional changes induced by exposure to space during development.
- Such research will provide vital fundamental information about how genetic and epigenetic factors interact with the environment to shape gravity dependent processes, and their penetrating influence across generations.
- Spaceflight experiments offer unique insights into the role of forces omnipresent on Earth (but absent in orbital flight) that can actively shape genomes in ways that are heritable.
- Such spaceflight experiments would place gravitational biology at the leading edge of modern developmental and evolutionary science.
- Ground-based studies should be conducted to develop specialized habitats to support reproducing and developing rodents in space.
- This research could be accomplished within 10 years.

Decadal Survey Priorities

“Mammalian reproduction is comprised of an intricate and complex series of events, including internal fertilization, implantation, placentation, organogenesis, fetal development, birth, lactation, parental care and postnatal maturation. Until the first mammal undergoes an entire life cycle in space, it is difficult to specify whether precise developmental phases are gravity-dependent. The minimal existing data, derived from rats, suggests that early reproductive events (i.e., fertilization, implantation, placentation, organogenesis), the transition from prenatal to postnatal life, and maternal-offspring interactions are high priority research areas. No mammal has given birth in space. It is unclear whether normal vaginal birth of rats or mice can occur in the space environment. Cesarean deliveries may be required.....”

“Lifespan studies of mammals to enable identification of gravity-dependent processes in the organization and development of the central nervous system and other organ systems, their integration, function and maintenance, and transmission across generations have not been accomplished. The small amount of existing data is derived from brief (<16 day) spaceflight studies with post-flight analyses at 1G. High priority studies include lifespan and multigenerational studies in the space environment incorporating developmental programming, epigenetics, and omics systems biology approaches during key reproductive and developmental phases in different model systems, especially mammalian models.”

“Developmental biology research is needed to fill gaps in our knowledge of the effects of the space environment on: (1) Gene-expression changes over time in model organisms, (2) DNA replication and repair processes, and their long-term consequences, (3) Changes in single nucleotide polymorphisms (SNPs) in large populations across multiple generations, (4) Replication and reproduction across multiple generations, (5) Intracellular molecular changes, (6) Changes in tissues, organs, physiological systems and whole organisms throughout the life span and across generations in space. Throughout these studies, it will be important to determine effects of gravity in relation to other space factors (e.g., radiation).”

Reproduction and Developmental Recommendations (rodents)

1. Studies should be conducted on transmission across generations of structural and functional changes induced by exposure to space during development.

•Such research will provide vital fundamental information about how genetic and epigenetic factors interact with the environment to shape gravity dependent processes, and their penetrating influence across subsequent generations. Spaceflight experiments offer unique insights into the role of forces omnipresent on Earth (but absent in orbital flight) that can actively shape genomes in ways that are heritable. Such spaceflight experiments would place gravitational biology at the leading edge of modern developmental and evolutionary science.

2. Spaceflight and ground-based (*e.g* centrifugation, hindlimb unloading) studies should be conducted to ascertain the role(s) of gravity in the organization, development and maintenance of the sensory and motor systems and their functional integration in mammalian systems.

- These studies will yield important new fundamental biology knowledge regarding the development and regulation of gravity-dependent brain, physiological and behavioral systems. High priority research should examine structural/functional changes in peripheral and central elements of the sensory and motor systems and their underlying mechanisms, critical periods of development, sensorimotor integration, neural plasticity and modulation of nerve growth factors, and adaptation.

3. Model systems offer increasingly valuable insights into basic biology. There should be a coordinated emphasis on utilization of invertebrates, non-mammalian vertebrates, and mammals to identify functional and evolutionary commonalities.

- Genetically altered models including mice should be used for analysis of key genes, gene products, and signaling pathways. This should include an organized effort to identify orthologous genes, common changes in gene expression, epigenetic analysis, and key model systems in space.

- Experimentation with rats should also be conducted to analyze gravity-based changes in certain systems (e.g., muscle) and to retain continuity with existing developmental space biology research.

Reference: *Recapturing a Future for Space Exploration: Life and Physical Sciences Research for a New Era*. Committee for the Decadal Survey on Biological and Physical Sciences in Space, National Research Council of the National Academies. The National Academies Press, 2011.

Reproductive Biology in Space – J. Tash

Cosmos 1129 Knowledge Gaps

- 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 later produced normal pregnancies and offspring (no developmental anomalies support epigenetic effect on sperm)
- No data available on follow-on assessment of females
 - Data cannot distinguish failed fertilization and no sign of pregnancies, from failure to mate (no video monitoring)
- Ground controls may have also failed to become pregnant
 - Hardware design issues?

Reproduction in Space Flight: Knowledge Gaps

- Whether mouse and rat reproductive factors respond similarly to space flight is unknown.
- Do males maintain normal spermatogenesis and produce normal sperm (including normal levels of DNA damage)?
- Do females continue to maintain normal oogenesis and produce normal numbers of normal oocytes (including normal levels of DNA damage)
- Do sperm capacitate (gain the ability to fertilize) normally?
- Do oocytes mature normally and is oocyte health maintained (in vitro and in vivo assessments)?
- Are there initial logistical and launch-related stressors that require recovery time (male and female)?
- Do any space flight induced declines in male and female reproduction recover post-flight?
- To date, the effects of spaceflight on male reproductive factors have been neglected.

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

- The three experiments used female C57BL/6 or BalbC mice.
- Microgravity negatively impacts ovarian histology in mice: 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 space flight (post-flight recovery has not been ascertained)
- Raises the hypothesis 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 space flight is unknown.

Male Reproductive Status in Hindlimb Unloaded Rats

- Long Term Hindlimb Unloading (HLS) Inhibits Spermatogenesis in Adult Male Rats in the Absence Of Cryptorchidism. All HLS animals were sterile in 2 mating attempts, controls were 100% fertile.
- Chronic testicular hyperthermia (elevated temperature: increased by 2.2°C, $P < .00001$)
- Invasion of inflammatory cells (≥ 3 weeks)
- Catastrophic apoptosis in the testes
- These factors cause aspermatogenic dysfunction

Behavioral Biology – J. Alberts (presented by A. Ronca)
Life Styles of Rats and Mice

- Less information is known for mice – much more known for rats.
- Commensal Habits of Norway rats (*Rattus norvegicus*) and House Mice (*Mus musculus*):
 - Rats: Live in colonies with multiple males and females. Promiscuous mating system – males mate with multiple females; females mate with multiple males.
 - Mice: Live in demes with 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 known for both maternal aggression, with which lactating dams vigorously defend nest and young against males, and for willingness to engage in “communal rearing” of young, with which they brood and nurse each others offspring along with their own.
- Colonial life of rats 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 associated with lower levels of tolerance among unrelated, unfamiliar adult males. Amicable contact behaviors are frequently seen among females but are not well understood.

Courtship and Copulation in Rats

- Females exhibit estrus cycle, 4 – 5 days continuously; some seasonality suspected but it's year-round in lab.
- Sexually “receptive” during phases of cycle, but they are more accurately defined as proceptive – because the estrus female actively solicits male's sexual attention.
- Female proceptive behavior includes
 - Moving into vicinity of male, making available their arousing, estrus odors, which attract male approach behavior and sniffing.
 - Female then “darts” away and stops
 - Male approaches again
 - Female darts and “ear wiggles”
 - The ‘dance’ continues and escalates until copulation

During copulation:

- Male mounts
- Female exhibits **lordosis**, making pudenda more accessible and intromission more likely
- Male may intromit and thrust
- Multiple intromissions and thrusts culminate in ejaculation

- Rats (and mice) are **reflex ovulators** in which the cervical stimulation from multiple intromissions triggers ovulation and makes fertilization possible
- 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 host of mechanisms and suspected adaptations related to copulation and reproductive success:
 - Odor-induced pregnancy blockades
 - Odor-induced resorption
 - Estrus synchrony (questionable by recent analyses)
 - 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 in form of copulatory moves, such as mounts, clasps, dismounts, and grooming sequences (known especially for mice).
- Surprising lack of information on interruptions of estrus cycle by general 'stress' factors; this would be worth investigating.
- Some data exist on habitat configuration (1-g) and patterned mating in rats, but comparable information not collected for mice.

Neurolab & Developmental Biology – D. Riley

Results and Lessons Learned from Neurolab

The Neurolab Mammalian Development Team of six principal investigators was formed by combining their individually-approved studies into a joint project. 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 groups. The initial assignment of litters as primary for specific investigators was discarded due to the high mortality in flight of PN8 pups that necessitated reapportioning to maximize science return.

The six experiments and major observations were:

- *Neural-Thyroid Interaction on Skeletal Isomyosin Expression – Baldwin*
Reductions in muscle growth, contractile protein expression and IGF-1R levels
- *Effects of Gravity on Postnatal Motor Development – Walton*
Persistent immature motor behavior, indicates missed critical period of gravity
- *Neural development under conditions of spaceflight – Kosik*
Hippocampal spatial navigation not impaired
- *Microgravity and Development of Vestibular Circuits – Raymond*
Reduced central neural interconnectivity, but peripheral sensory organ intact
- *Development of the Aortic Baroreflex under Conditions of Microgravity – Shimizu*
Transient low baroreceptor sensitivity and weak vascular contraction
- *Effects of Microgravity on Neuromuscular Development – Riley*
Delayed maturation of fiber types, motoneurons and neuromuscular junctions

The effects of spaceflight on development ranged from not impaired 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, and the flight duration of 16-day gravity deprivation was likely too brief to have a major impact on some systems.

Expected influences of spaceflight

- Based on flight and ground studies to date, severe and potentially permanent disruptions of development, growth, maturation and aging of the nervous and musculoskeletal systems are anticipated when the life cycles of rats and mice are spent totally in the spaceflight environment.
- The absence of gravity is expected to cause improper formation and maintenance of normal (one-gravity) patterns of neural connectivity in the brain, spinal cord and peripheral nervous systems.

- Another serious risk, not limited to the nervous and musculoskeletal systems, is the predicted inadequate generation of stem cells, such as myoblasts, osteoblasts, osteoclasts, which are essential for normal development of muscles and bones and enable lifetime adaptation and repair to preserve optimal function and avoid premature aging deterioration.
- During long-term spaceflight, rapidly dividing cells (such as progenitor cells) are especially vulnerable to radiation-induced DNA damage with negative consequences for the tissues derived from the mutated cells, leading to abnormal and cancerous phenotypes.

Lessons Learned from Neurolab (16-day mission)

- 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 (one-gravity) 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., multiple generations in space)
- Return of live space-reared organisms is necessary for assessment of exposure to one-gravity 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 one-gravity 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.

Key Follow-up Questions

- Does the absence of one-gravity in a specific time window of development disrupt normal development, maturation, and aging of the musculoskeletal and nervous systems?
- Are the spaceflight-induced developmental and growth abnormalities permanent, implying the existence of critical periods of gravity-dependent processes?
- In adult rodents exposed to long term spaceflight of three or more months, what are the rates of bone and muscle adaptation to the spaceflight environment, and what are the steady state endpoints of deterioration?
- What is the nature and severity of the damage and degree of repair when rodents that are fully adapted to the spaceflight environment are returned to one-gravity to complete their life cycle?

Alternate Platforms including Chronic Acceleration & Hindlimb Unloading (HLU) – C. Fuller

Chronic Acceleration

- Resultant of 1g Earth gravity and centrifugal force. One such experimental facility is the UC Davis Chronic Acceleration Research Unit.
- Only produces hypergravity environment compared to starting conditions
 - On earth > 1G
 - In Space > 0G
- Considerations
 - Symmetrical loading of entire organism
- Altered signaling of CNS sensory input
 - Principle of gravitational continuum (Wade, Advances in Space Biology and Medicine, 2005, 10:225)
 - Acute or Chronic exposure
- Indefinite period of exposure
- Husbandry limitations

Hindlimb Suspension

- Unloading support structures
 - Bone
 - Muscle
- Cephalic fluid shifts
- Mimicking spaceflight effects for some systems
- Considerations
 - Immobility
 - No loss or alteration of CNS sensory input
 - Duration of exposure

VI. Notes from Discussion on Breeding, Birthing, and Postnatal Development in Space

Overview of Discussion (adapted from J. Alberts)

Major Goals	<u>Breeding</u> : Visual evidence of paced mating behavior, including solicitations by females, approaches by males, mounting, intromissions, and ejaculation. Evidence of implantation, placentation, gestation.	<u>Birthing</u> : Females survive parturition, establish nursing behavior, and maintain contact with entire litter of offspring. Offspring survive in numbers equivalent to ground controls and suckle successfully for 4 days (“initiation” phase of maternal behavior cycle).	<u>Nursing/Weaning</u> : Nursing behavior continues through weaning of young; dam maintains energy balance while lactating. Offspring achieve adequate growth rates to support sensory, motor, and morphological development appropriate for successful weaning and achievement of puberty.
Mice or Rats - which species offers the best chance to meet the goals?	<u>Breeding</u> : Rats. The literature is more detailed and complete on rat courtship and copulation than on mice. Also, more flight heritage (Sprague-Dawley) and knowledge base with rats. In mice, there seems to be more inter-strain differences in behavioral parameters for mouse breeding, including cases of resorption and pregnancy block.	<u>Birthing</u> : Rats. Mice may be more susceptible than rats to cannibalizing or abandoning young litters if their nests are disrupted, especially if the disruptions occur early – during the initiation phase of maternal behavior.	<u>Nursing/Weaning</u> : Rats. Lactation and mouse-pup growth are more vulnerable for mice than in rats. There are probably more sources of interference or possible failures for mice than for rats.

Pair or Harem Breeding? Harem, i.e., more than one female per male, for both species. For rats, a minimum harem-unit of 2 females : 1 male is the standard in the field. An alternate harem-unit would be 3 females : 1 male.

It is suggested that 4 harem-units be flown in a validation flight, with two habitats each subdivided to accommodate 2 harem-units per habitat. At the minimum ratio, this could yield up to 8 litters.

Male and Female Breeding History Experienced breeders and proven mothers should be used. Ideally, proven females *and* proven males will be used, and preferably proven *harems* or *couples* will be used.

Special Considerations

- Habitat shall support (or be adaptable to support) all phases of life cycle: birth to weanlings to 3 months and 6 months; e.g., internal cage modules might be changed to better suit different phases of the life cycle. Habitat shall support each stage of reproduction and life of neonates. Habitat support of birthing and of neonates should be validated on ground prior to space experiments. Mouse and rat mating in habitats should be proven on the ground prior to space experiments.
- Enrichment or nesting material should be considered for inclusion in the habitat. Active nest building is a milestone along the way to conception and a characteristic of maternal behavior.
- Continuous video is required (for behavior). Quality (resolution) and position of cameras TBD.
- Video monitoring will be more heavily used in Reproductive/Development than other rodent experiments.
- The infrared camera should be recording continuously (24 hours a day, 7 days a week). The visible camera and visible light should only be on during the light cycle.
- During launch, separate the males from the females until microgravity has been achieved, and, potentially, while the animals are acclimating to weightlessness. Afterwards, put harem-units together for attempted mating. Then, remove males prior to the time of birth.
- House females together prior to introducing males in order to synchronize the estrous cycle.
- Live-animal return from ISS is required to test subsequent breeding and birthing potential of animals that were unsuccessful breeders during space flight.
- Strain-specific databases may need to be generated in ground testing and flight experiments in order to establish flight selection criteria.
- Preferred rat strain: Sprague-Dawley, as they have the most flight heritage and the largest body of knowledge for reproductive and development research.
 - Alternate rat strain: Fisher 344 rats were used for Neurolab.
 - Alternate rat strain: Long-Evans rats are used in male contraceptive research. Females of this strain show maternal behavior.
- Albino rat eyes (e.g., Sprague-Dawley) may show exacerbated retinal degeneration due to luminosity of habitat lighting. This should be considered and tested during the habitat design.
- If launch forces and vibrations are concerns, consider training rodents on shaker table and centrifuge.
- Utilize a stepwise approach to study reproduction and development. Obtain science data at multiple stages of reproduction and development.
- Range of the relative humidity in the habitat is uncertain. What is the likelihood of reaching the lower acceptable bound of 30%? What is the relative humidity in the neonate huddle? Ground testing should be performed to define these parameters.

Summary of Major Risks and Mitigation Approaches

Breeding: Question of whether females will cycle hormonally in novel environment of spaceflight? If females cycle, will they solicit and will males respond? In microgravity environment, can pairs manage mechanics of repeated intromissions needed for ejaculation and reflex ovulation?

Mitigation of Breeding Risks: Use proven breeders. Cage design should incorporate the biomechanics of mating. Test specific strains of each species in 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 species. Disrupted onset and initiation of maternal behavior cycle is a major risk (unaddressed by Neurolab experiences). 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: 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 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 reasonable to expect. Dam approaches unified group of pups for first 12-14 days postpartum. Important because all nipples get milk at once per letdown and thus all pups should be attached to get fair share. Neurolab showed us 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.

Problems Concerns, Risks, and Knowledge Gaps from the Discussion on Breeding, Birthing, and Postnatal Development in Space

Subcategory	Basic Science (S) / Hardware (H) / Enabling Science (ES)	Risk (R) / Concern (C) / Gap (G)	Priority	Concerns, Risks, & Knowledge Gaps	Risk Mitigation Approaches
Cross-cutting issues	S	G, R	Low	What are the changes in endocrine status, including HPG axis and prolactin during spaceflight?	
	S	G, R	Low	Are there fertilization issues during spaceflight?	Artificial Insemination
	S	G	Low	Does the elevated radiation level affect reproduction or fetal/neonatal development?	Localized dosimetry at or near (or that closely resembles the shielding characteristics of) the habitat and any exposure from DXA machine.
	H, ES	C, G	High	Information is needed to enable the understanding of behavior and of experiment failures.	Video and monitoring of key parameters
	H, ES	C, G	High	Does the environment (i.e., air circulation, noise) within the habitat impede acoustic and olfactory communication between neonates and dam?	Define and measure indices of survivability and thrivability
	H	C, G	High	Required crew access to animals should inform cage design and procedures (science, safety, etc), see SRED.	
	H	C	High	Cage design will play an important role in promoting or inhibiting success of all categories of reproduction and development.	Wall surface texture and grip; dimensions of birthing space; space for the young to huddle and suckle; temperature control
	ES	C, G	High	Does the current diet present any nutritional deficiencies impeding aspects of reproduction?	Pair feeding. Measure food and water intake.
	ES	C, G	High	What are the enrichment requirements for Breeders?	
	ES	G	High	What is the acclimation period before haremns are ready to mate?	Generate data from a Mark I experiment.
	ES	C, G	Med	How long have prior foodbar tests/studies been performed? Are there any foodbar inadequacy or stability issues with long-term use of the foodbar?	
	ES	C	Low	Most of the knowledge is from studies using rats. Issues can emerge from extrapolation to mice.	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.
Mating Behavior	S	G, R	Med	Is copulation affected by space flight?	Selection of proven breeders or breeding pairs
	S	G, R	Med	Is estrous cycling altered by space flight?	
	H, ES	G, R	High	Are pre-mating behaviors and courtship affected by space flight or the habitat design?	Selection of proven breeders or breeding pairs
Lactation	S	G, R	Med	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	G, R	Med	Is the quality and quantity of milk altered during space flight?	Pre- vs. during- flight comparison
	S	G, R	Med	Do changes in immune function (antibody status in milk; absorption) affect lactation?	
	H, S	G, R	Med	Are nursing behaviors of the dam and pup, including suckling and retrieving, altered during space flight?	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).
	ES	C, G	High	Is the foodbar adequate for lactation?	

Problems Concerns, Risks, and Knowledge Gaps from the Discussion on Breeding, Birthing, and Postnatal Development in Space

Subcategory	Basic Science (S) / Hardware (H) / Enabling Science (ES)	Risk (R) / Concern (C) / Gap (G)	Priority	Concerns, Risks, & Knowledge Gaps	Risk Mitigation Approaches
Pre-Fertilization	S	G, R	High	Are gonad and gametogenesis and their functions compromised during space flight?	
	S	G, R	High	Is the endocrine status, including HPG axis, altered by spaceflight environment?	
	S	G, R	Med	Are post-gonad gamete formation, function, and maturation compromised?	
Fertilization	S, ES	G	Med	Does the current diet present any nutritional deficiencies impeding aspects of fertilization?	Pair feeding. Measure food and water intake.
	S	G, R	High	Are sperm-egg signaling and interactions compromised?	
	S	G, R	High	Is pre-implantation development compromised in the dam?	Consider results of STS-131, -133, -135 for underlying mechanisms in mouse.
	S	G, R	Med	Is signaling in the fertilized egg compromised by mechanisms including altered gene regulation and DNA damage?	
Implantation	S	G, R	High	Does mating trigger proper signaling to prepare uterine epithelium for implantation?	
	S	G, R	Med	Are adhesion and implantation gravity dependent?	
	S	G, R	Med	Are uterine epithelial health and stem cells compromised?	
Placentation	S	G, R	Med	Does decreased connexin-43 levels affect placentation during spaceflight?	
	S	G, R	Med	During spaceflight, does placentation depend on changes in vascular tone?	
	S	G, R	Med	Are the signals for placental formation intact?	
	S	G, R	Low	During spaceflight, does placentation depend on changes in the immune system?	
	S	G, R	Low	What are the statuses of prolactins and other pro-placentation endocrine signals during spaceflight?	
Organogenesis	S	G, R	Med	Are organ formation, maturation, and function compromised during spaceflight?	
	S	G, R	Low	Are the endocrine-driven fetal-development phases that define the sex of offspring altered during spaceflight?	
Birth (also see cross-cutting)	S	R	Low	Will reduced strength in abdominal musculature affect birth?	Caesarean section
	S	C, R	Low	Will altered uterine contraction strength affect birth?	Cage design - wall surface texture and grip; dimensions of birthing space
	S	G, R	Low	Does the likelihood of a successful birth depend on genetic strain of rat?	
	H, S	G, R	Low	Is the maternal care pattern at birth intact?	1 hour of pup cooling - temperature regulation; Video and temperature monitoring; space for the young

Problems Concerns, Risks, and Knowledge Gaps from the Discussion on Breeding, Birthing, and Postnatal Development in Space

Subcategory	Basic Science (S)/ Hardware (H) / Enabling Science (ES)	Risk (R) / Concern (C) / Gap (G)	Priority	Concerns, Risks, & Knowledge Gaps	Risk Mitigation Approaches
	ES	C	Med	With regards to testosterone formation, the temperature exposure of male pups should be closely monitored and potentially regulated.	Include capability for thermography of the pups within the habitat.
Perinatal Development (PND 0-8)					
	H, S	G, R	Med	Are the pups receiving milk in similar manner as ground controls?	Observing through skin: milk bands (acquiring milk); tooth eruption. Gauging milk-let downs through video
	H, ES	G	Med	Is the amount or quality of sleep that the neonates receive affected by spaceflight or cage design?	Video
	H, ES	C	High	In case of cannibalism, how to quantify the number of pups that were initially born?	Video. Count # that are born. Higher frequency of monitoring around key milestones.
	ES	C	High	How will individual pups be identified for further monitoring?	Ink in footpad or tail tattoo/markings, toe-clipping. Crew access. ANG; coat color.
	ES	C	Med	Body mass shall be recorded 2x/week.	Repeated measurement of body mass is important during the first few weeks post-natal to assess growth.
Infant Development (PND 8-14)					
	S	G	Low	Do changes in the development of ovaries and testes occur during spaceflight?	
	H, S	G, C	Med	Is the amount or quality of sleep that the neonates receive affected by spaceflight or cage design?	Video
	H, ES	C	High	Huddle formation needs to be considered during cage design, as it is an integral aspect of neonatal development.	Cage design (artificial nest)
	H, ES	G	High	Does the relative humidity need to be regulated within a specific range for neonates?	
	H, ES	G	Med	Are there thermal-regulation requirement differences between pups and mother?	Cage design (compartments with unique thermal regulation). Thermography capability needed.
Pre-Weaning (PND 15-21)					
	S	G	Low	Do changes in the development of ovaries and testes occur during spaceflight?	
	S	C	Low	Milestone: Independent ingestion should occur around PND15 (Neurolab).	
Adolescence (PND 28-35)					
	ES	C, R	Low	Consider the risk of impregnation of siblings at sexual maturity when establishing age to separate the litter.	Male-female separation Mice: approx. 21-35 days Rats: approx. 45-80 days

Ground-Based Studies to Address Knowledge Gaps

- Rodent habitat biocompatibility testing of reproduction, rearing, and growth for a total of at least 3 months
- Assess, and upgrade as needed, the quality and adequacy of video and still photography (~1/5 sec) to meet the scientific requirements to document animal behaviors (activity monitoring, limb functions, sleep adequacy, pup-pup and pup-dam interactions) in the habitat
- Return logistics – specimen and live animal return operations, including landing site animal observations and animal processing and transportation and processing at land-based sites
- In females and males, compare the processes of reproduction and gestation in rats and mice in response to simulated spaceflight (e.g., hindlimb unloading)
- Studies of rat and mouse birth and early neonatal development to determine optimal nest conditions (e.g., size, bedding, surface temperatures, and chamber temperature and their effects on the temperature of the neonatal huddle) to maximize growth and development of offspring.
- Behavioral studies of harem mating in rats and mice to determine factors that will optimize reproductive success

VII. Top-Level Science Requirements For the Mark III Habitat – R. Globus

Yellow highlights indicate additions or modifications to the SRED, Rev. E.

3.0 EXPERIMENTAL DETAILS AND SCIENCE ENVELOPE

• **Table 1**, below, provides guidance for science disciplines (rows) and subject and experiment requirements (columns). The rodent subject requirements are categorized as number, species, gender and age. Also included are comments on ground control numbers and live return requirements.

3.0.1 NUMBERS

- REQUIREMENT: Rodent specimens shall be provided for on orbit and ground research at the numbers specified in Table 1

3.0.2 SPECIES

- REQUIREMENT: Habitats shall accommodate mice or rats as specified in Table 1.

3.0.3 GENDER

- REQUIREMENT: Habitats shall accommodate male and/or female specimens as specified in Table 1.

3.0.4 STRAIN

- REQUIREMENT: Habitats shall accommodate rodents of different strains as specified in Table 1.

3.0.5 ANIMAL AGE

- REQUIREMENT: Habitats shall accommodate rodents of all ages and phases of the life cycle specified in Table 1.

Alternatively, multiple habitat configurations, designed to specifically accommodate specific phases of the rodent life-cycle (for purposes of reproduction and developmental studies), shall be developed.

3.0.6 DURATION

- REQUIREMENT: Habitats shall provide: adequate housing for rodents during transport and crew access for treatment and/or removal of animals for in-flight procedures to clean habitats for an on-orbit operational capability of up to 180 days on ISS.

3.0.7 LIVE ANIMAL RETURN

- REQUIREMENT: Return of live rodents post-flight to a site where animals can be handled and tissue recovered shall be accomplished within six hours post-landing.

3.1 Animal health maintenance requirements

- REQUIREMENT: Animals shall be maintained in a healthy state, suitable for scientific inquiry into the influence of spaceflight.

• REQUIREMENT: Habitats shall support all phases of the rodent life cycle. Alternatively, a separate habitat designed to specifically accommodate rodents for purposes of reproduction and developmental studies shall be developed.

3.1.1 Pre-flight

- REQUIREMENT: Animals shall be maintained in AAALAC-accredited animal care facilities, and all live animal transport and activities between facilities shall be pre-approved by a NASA IACUC. Unless otherwise requested by project and science teams, animals shall be transported to the launch site animal care facility three weeks before launch.

3.1.2 Ground controls

- REQUIREMENT: Ground control animals from the same cohort as flight animals shall be housed in habitats matched to flight units; environmental conditions for ground controls shall be controlled to match those of flight animals unless otherwise requested by investigators and/or the project team.

3.2. Specimen Treatments

3.2.1 Specimen and Treatment Requirements (Pre-flight)

- REQUIREMENT: Habitats shall support animals that have been administered various pre-flight treatments including acclimation, drug implants, injections and other experiment-specific operations.

3.2.2 Specimen and Treatment Requirements (In-flight)

3.2.2.1 REQUIREMENT: Animal health shall be monitored daily by direct (crew observation) or indirect (video recording and interpretation by a veterinarian or his/her designee) methods; food and water intake shall be monitored to confirm adequate supply and continued consumption.

SUB-REQUIREMENT: For reproductive and development studies (Mark III), video recording and thermography shall be performed 24 hours per day/7 days per week during TBD periods of the life cycle.

3.2.2.2 REQUIREMENT: On-orbit operations shall be performed in support of future science objectives per Table 3. These operations include but are not limited to injections, anesthesia, euthanasia, gross dissection, tissue collection, sample analyses on ISS, cell culture and optimal sample preservation for post-flight studies.

3.2.3 Specimen and Treatment Requirements (Preservation and Post-Flight Analyses)

- **REQUIREMENT:** Rodent specimens shall be maintained at temperatures, consistent with Table 2 (throughout post-flight recovery, transport and distribution based on requirements of sample handling and analysis).

3.3 FUTURE INVESTIGATIONAL AREAS (see Table 1)

3.3.1 Long Duration

3.3.2 Reproduction, Development and Multi-generational studies

- **REQUIREMENT:** **Habitats** shall be provided that support animals throughout their lifespan, i.e., birth and nursing, sexual maturation, reproduction, and aging.

3.3.3 Centrifugation: Fractional Gravity studies: To be covered in a separate SRED

Table 1: Representative Experiment Details*

Science Disciplines: Future Increments	Specimen	Strains	Flight Mice (Rats) numbers/flight	Gender	Age** (weeks)	Duration on ISS (days)	Ground controls (housed in flight-like hardware)	Live Animal Return - Mice (Rats)
Requirement	3.0.2	3.0.4	3.0.1	3.0.3	3.0.5	3.0.6	3.1.2	3.0.7
Immune system	Mice	Tbd	20-40 (10-20)	F (or M)	≥8	15 to 90	Yes	10-20 (5-10)
Bone	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	>12	30 to 90	Yes	10-20 (5-10)
Muscle	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	≥8	30 to 90	Yes	10-20 (5-10)
Neuro-sensory/ Vestibular	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	≥8	15 to 90	Yes	10-20 (5-10)
Cardiovascular/ Respiratory	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	≥8	15 to 90	Yes	10-20 (5-10)
Metabolism/ Endocrine	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	≥8	30 to 90	Yes	10-20 (5-10)
Long duration studies: All of the above	Mice (or Rats)	Tbd	20-40 (10-20)	F (or M)	≥8	180	Yes	10-20 (5-10)
Mating / Reproduction / Development NOTE: (mice) are in parentheses.	Rats (mice)	Tbd	12 rats (4 harem-units @ 2 females and 1 male per harem-unit.) (12 offspring per dam, with 10 offspring carried forward.) (10-20)	F and M	0-26	10-210 days	Yes	5-10 (10-20)
Multi-generation (F0 to F2/F3) NOTE: (mice) are in parentheses.	Rats (mice)	Tbd	12 rats (4 harem-units @ 2 females and 1 male per harem-unit.) (12 offspring per dam, with 10 offspring carried forward.) (10-20)	F and M	0-26	33 - 42 weeks	Yes	5-10 (10-20)
CASIS Initial priority is Bone/Muscle	Mice (or Rats)	tbd	20-40	F (or M)	>12 weeks	30-90	Yes	TBD

Key: Yellow highlights indicate additions or modifications to the SRED, Rev. E.

* Reproductive and aging studies expand the envelope from birth to old age, e.g. 1.5-2 yrs

** An in-flight duration <30d may be requested by specific PIs.

***Some experiments may entail post-flight processing of live animals to begin later than 6 hours post-landing.

Table 2: Animal health maintenance requirements – Mark III

Parameters	Requirement Values	Monitored	Downlinked	Real-time data	Comments
O ₂	20 % + 10 / - 4	Yes	Yes	Yes	Values assume 1 atm pressure; Monitor at site reflecting conditions within habitat.
CO ₂	≤ 6000 ppm averaged over 24 hr or ≤ 7000 ppm at any given time	Yes	Yes	Yes	SMAC (Spacecraft Maximum Allowable Concentration) is 7000 ppm. Monitor at site reflecting conditions within habitat.
Ammonia	Less than 25 ppm	TBD	TBD		Periodic sampling shall be conducted in select ground verification tests
Humidity	Post-weaning: 30-70% * Neonates: TBD	Yes	Yes	Yes	40-60% measured as typical vivarium. Guidelines go down to 30%. Monitor at site reflecting conditions within habitat.
Temperature	Surfaces in contact with animals Post-weaning: Not exceed 37 C Neonates: TBD	No	No	No	Post-weaning: Establish surface temperature during operation while conducting select ground verification tests.
	Chamber Post-weaning: 20-26 C Neonatal Nest: Optimal 33 C, with range TBD	Yes Yes	Yes Yes	Yes Yes	Post-weaning: Previous flight experiments in range of 24-33 °C determined acceptable.
Acceleration / Vibration	Launch	TBD	Yes	No	Pending from Bion data (launch: April 19, 2013) and/or Space-X data.
	On-Orbit	TBD	Yes	No	Monitor at site reflecting conditions within habitat. (Cabin or rack data acceptable).
	Re-entry		Yes	No	For live animal return, monitoring of acceleration shall continue until termination of recovery and transport operations.

Table 2: Animal health maintenance requirements – Mark III

Acoustics					Short-duration exposure limits during launching and re-entry: TBD (Middeck reference value: 117.5 dB OASPL)
• Launch (SpX)	TBD	Yes	No		
• On-Orbit (ISS)	84.81 dB OASPL	Yes	No		Cabin or rack data acceptable
• Re-entry (SpX)	TBD	Yes	No		
Air Velocity	<p>Post-weaning: 0.1-0.3 m/s (± 0.01 m/s)</p> <p>Neonates: TBD</p>			Yes	<p>Post-weaning: As measured in an empty specimen chamber. Measured per minute. No less than 10 air volume changes per hour.</p> <p>Differences within Specific Compartments: TBD.</p>

Key: Yellow highlights indicate additions or modifications to the SRED, Rev. E.

*Source: Guide For Animal Care and Use of Laboratory Animals, 8th edition. National Research Council. 2011. Washington, DC: National Academies Press.

Table 3: On-orbit operations (Note: details pending further review by SWG/CASIS)

In-flight Operations	Immune systems	Bone	Muscle	Neuro/ Sensory/ Vestibular	Cardiovascular/ Respiratory	Metabolic/ Endocrine	CASIS	Repro/Dev. Multi Gen	In-flight Requirements Mice (rats)
Injection	x	x	x	x	x	x	x	x	Mice: <0.5 ml Rats: <1.0 ml
Body mass measurement	x	x	x	x	x	x	x	x	Mice: 5 –50 g Rats: up to 1000 g
Anesthesia	x	x	x	x	x	x	x	x	Varies; Sterile injection of <0.5 ml (mice); 1.0 ml (rats)
Dissection*	x	x	x	x	x	x	x	x	Use of MSG
< Euthanasia	x	x	x	x	x	x	x	x	mice<0.5 ml rats:<1.0 ml
< Densitometry		x	x				x	x	Multiple measurements per experimental duration
< Blood draw	x	x	x	x	x	x	x	x	TBD
< Surgery (survival)	x	x	x	x	x	x		x	Monitoring stress and body temperature during recovery post-op
Sample / carcass storage									+2-4 °C for fixatives -95 °C for structural tissues -180 °C for cells
< +2 C									
< -95 C	x	x	x	x	x	x	x	x	
< -180 C									
< ISS Ambient									
< Freezing (Fast)**	x	x	x	x	x	x	x	x	TBD
< Snap freezing	x	x	x	x	x	x	x	x	TBD
< Freezing (Viable Cell-Controlled)	x	x	x	x	x	x		x	cell freezing holders; 1C/min to -80°C; then transfer to -180 °C

Table 3: On-orbit operations (Note: details pending further review by SWG/CASIS)

In-flight Operations	Immune systems	Bone	Muscle	Neuro/ Sensory/ Vestibular	Cardiovascular/ Respiratory	Metabolic/ Endocrine	CASIS	Repro/Dev. Multi Gen	In-flight Requirements Mice (rats)
Cell Culture (Ex-Vivo)	x	x							37 °C environment with 5% CO ₂
Gene Express'n qRT PCR (in-flight)	x	x	x	x	x	x	x	x	RT-qPCR system - including cell/tissue homogenizer, centrifuge and PCR with reagents; downlink data capability required
Biotelemetry			x	x	x	x	TBD	x	
Thermography (with downlink)	x					x		x	Continuous video TBD for Repro/Dev
Organ/Tissue Function	x	x	x	x	x	x	x	x	
Behavior/Performance (video with downlink)				x		x		x	Continuous video TBD for Repro/Dev
Post- flight Sample Analysis									
Proteomics	x	x	x	x	x	x	x	x	
Genomics	x	x	x	x	x	x	x	x	
Metabolites	x	x	x	x	x	x	x	x	
Structure	x	x	x	x	x	x	x	x	

Key: Yellow highlights indicate additions or modifications to the SRED, Rev. E.

* A dissection microscope or other magnification device may be needed for some specific dissection and surgical procedures for some experiments.

**Carcasses (or any part/tissue thereof) shall be placed in a freezing environment within 30 min of death or immediately after termination of dissection, or other procedure such as bone densitometry, etc. Carcasses shall remain frozen continuously until recovery on Earth.

VIII. Examples of Relevant Flight Experiments

Reproductive Biology – J. Tash

- Multigenerational survival comprises a cycle of lifespan functional milestones at the multiple levels: whole animal, organ & endocrine systems, and cell (Figure 2). A failure or deficit in any milestone compromises species survival
- Male and female sexual definition and development:
 - Gonad development, maturation & health
 - Gamete production, maturation & health
- Social behavior and mating:
 - Ex-gonad gamete maturation in the female reproductive tract
 - Fertilization, conceptus, placentation, fetal development
- Support of pregnancy, birth, lactation, nursing, weaning
- Post-weaning growth, puberty, acquisition of sexual maturity

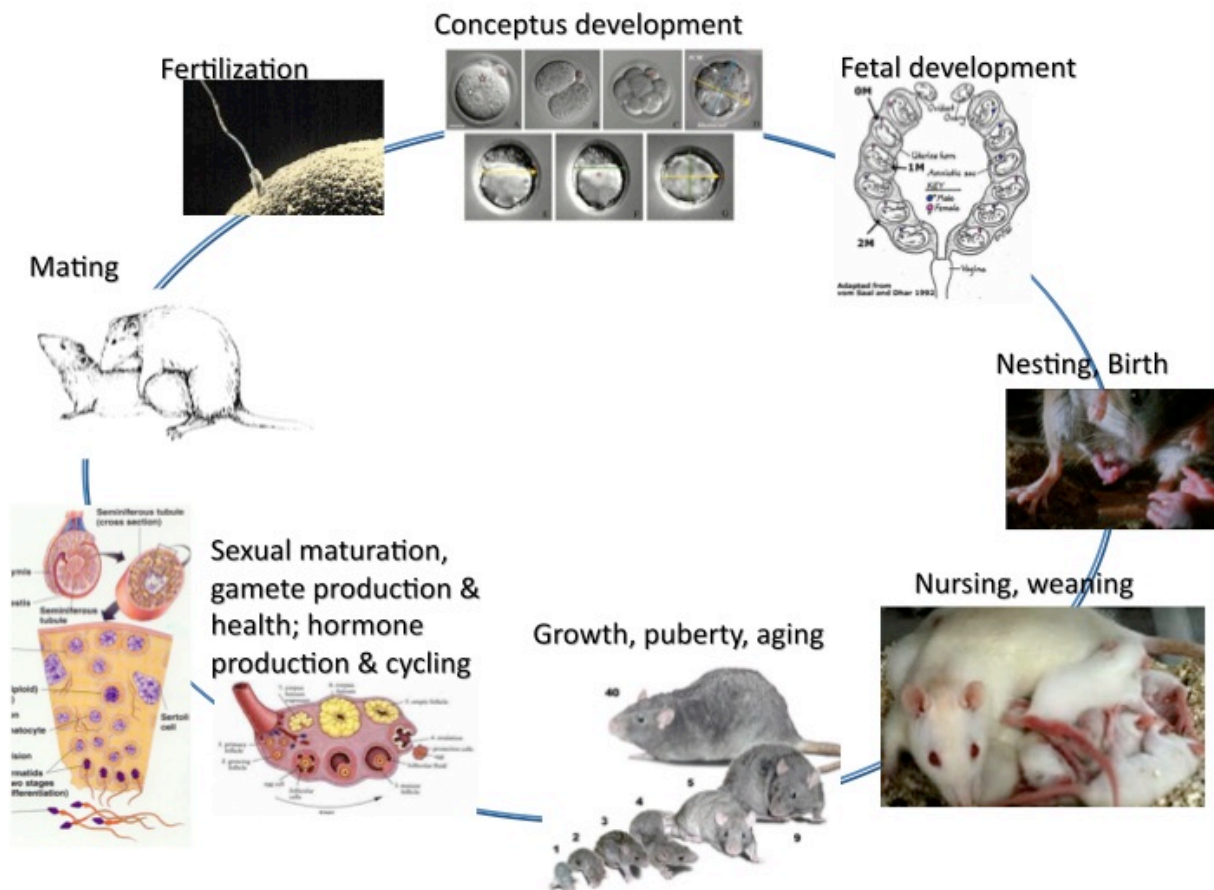


Figure 2 Multigenerational Success is a Repeating Cycle of Necessary Milestones

Neuroscience and Behavior – A. Ronca

1. Is the neural architecture of the brain, particularly the gravity sensing system, shaped by gravity?

Hypothesis: Neuronal connectivity within the neurovestibular system is reduced in rodents reared in microgravity.

2. Are vestibular mediated behaviors shaped by gravity?

Hypothesis: Behavioral responses that rely upon gravitational sensory input will be reduced in rodents reared in microgravity and responses correlated with patterns of neuronal connectivity.

3. Is development of the motor system dependent upon gravitational input?

Hypothesis: The emergence of fine motor control of locomotion and gait require gravitational input during development.

4. Are there critical periods during which gravity exerts formative effects?

Hypothesis: The critical period for gravity's formative effects span the prenatal and postnatal periods.

5. 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?

Hypothesis: Growth and development, metabolism, the circadian timing system and phenotypic behavior of WT mice exposed to microgravity will be comparable to that of genetically altered mice with macular deafferentation.

6. Is there epigenetic (non-genomic) cross-generational heritability of early life programming by gravity?

Hypothesis: Identifiable epigenetic changes in DNA methylation patterns will occur in response to development in microgravity (a direct effect) distinct from indirect maternal contributions to epigenetic programming of off spring phenotype.

Musculoskeletal – D. Riley

For animals born in space and undergoing development, growth and aging are there critical periods of development and growth that require gravity?

- *on-orbit, temporal, noninvasive bone density and shape measurements
- *on-orbit, temporal measurements of body weight to assess growth
- *on-orbit, temporal muscle diameter and length measurements
- *on-orbit tissue acquisition and preservation of bone, cartilage, and muscle at key time points. Intact sample return for earth-based analyses

Is stem cell production (myoblasts, osteoblasts, osteoclasts, bone marrow cells) reduced during prolonged spaceflight?

- *quantify stem cell populations using cell specific type markers
- *return rats to one-gravity to evaluate reloading injury and the capacity for repair that is dependent on stem cell participation
- *determine whether re-exposure to one-gravity restores stem cell deficiency or reveals permanent deficiencies

General Physiology & Immunology – C. Fuller

- General Physiology & Immunology
- Overarching foci:
 - Altered regulation (System to Genome)
 - Adaptive capabilities (i.e., 0->1G)
- CNS Function
 - Covered by Neuroscience & Behavior Group
 - Role in altered sensory signaling, esp. Central (Vestibular) sensing, in altering genome
- Musculoskeletal System
 - Covered by Musculoskeletal Group
- Growth, Body Size and Composition
 - Gender
 - Health
- Endocrinology
- Metabolism
 - Energetics
 - Nutrition
 - Gastrointestinal
- Cardiovascular & Blood (i.e., Oxygen transport)
- Temperature Regulation
- Circadian Biology
- Immune Function
- External Stimuli:
 - Exercise
 - Centrifugation

Commercial – L. Stodieck

- *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, etc.).
- *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.

IX. Workshop Presentation Summaries

Rodent Habitat and AEM-T and -X Overview – M. Hines

- Rodent Habitat Project includes of a suite of hardware developed by ARC
 - Rodent Habitat – provides on-orbit housing for rodents in an ExPRESS rack
 - Animal Transport System (ATS)
- Transporter – provides housing for rodents during ascent on Dragon
- Life Support (BioServe) – provides Environmental Control and Life Support (ECLS) for rodents during ascent on Dragon (CO₂ scrubbing, O₂ supply, moisture control)
 - Animal Access Unit (AAU)– Interfaces with both the Rodent Habitat and the Transporter for transfer of the animals between the units and access to the animals for science operations
 - Hardware Support Kits
- foodbars, clean up, and containment bags
 - Experiment Unique Equipment
- Specific to each flight based on science requirements

Animal Enclosure Module-X (AEM-X) and -Transfer (T)

- Pictured in Figures 3 and 4, respectively
 - Single MLE unit houses 10 mice or 3 – 6 rats
 - Front breather
 - Temperature and RH monitoring, no active thermal control
 - Visual measurement of food & water
 - Transfer animals to a clean Rodent Habitat with a full complement of food and water after 20 - TBD days to achieve longer duration missions
 - Improved science and animal husbandry through video monitoring and in-flight access
 - 2014 – fly Rodent Habitat – adapted to operate at the ISS/EXPRESS Rack
 - Launches and returns as passive stowage on Dragon
 - Powered only during duration of experiment operations
-
- Blowers & exhaust filter in rear (air speed 0.15 m/s)
 - Visible and IR video
 - LED lighting (white (40 lux, mid-cage) and IR)
 - Temperature and humidity sensors
 - Status, sensor, and video downlink capability
 - Commanding capability
 - 45 dBA acoustic noise level (exterior)

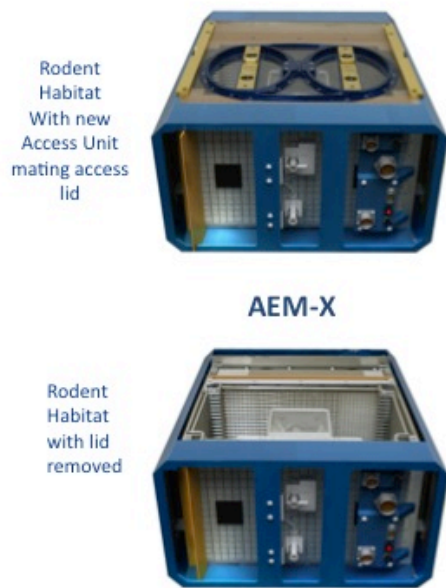


Figure 3 AEM-X will house up to 10 mice (6 rats) on board ISS.

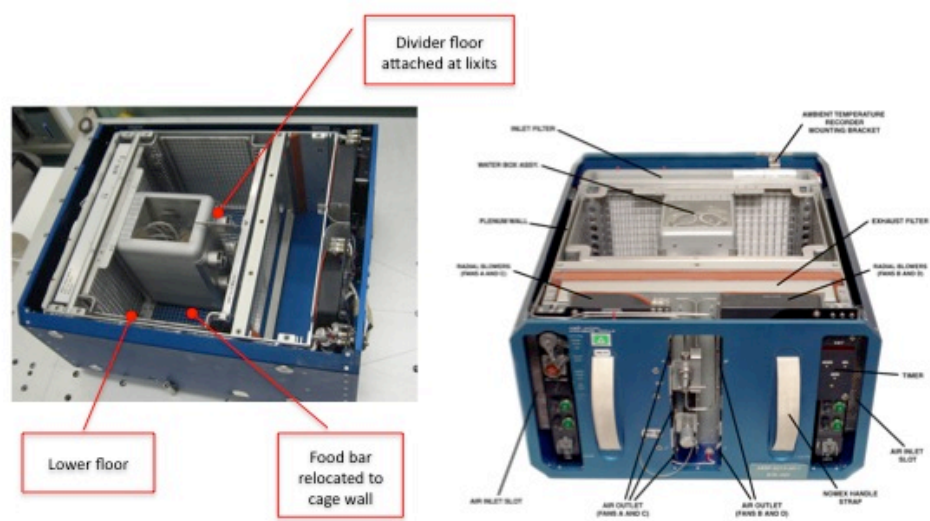
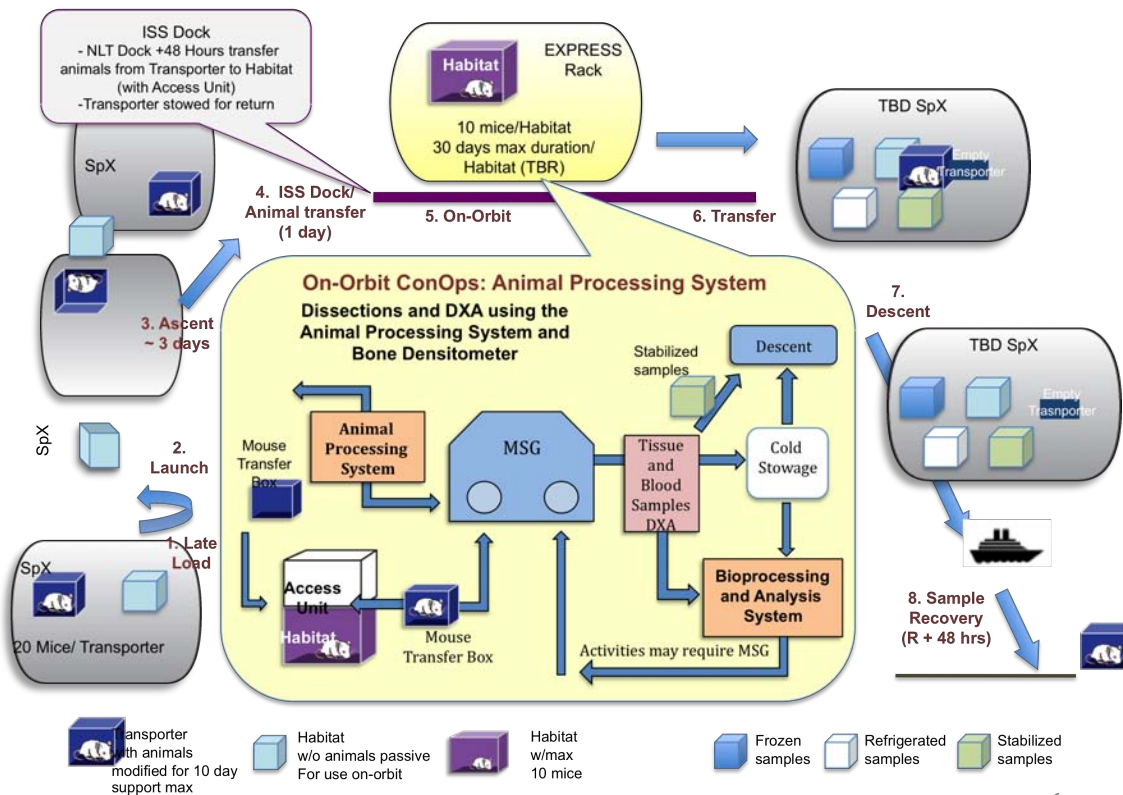


Figure 4 AEM-T is derived from the original AEM and houses 20 mice on board SpaceX Dragon while in transit to ISS.

ISS Mission Characteristics: Launch, Concept of Operations, Recovery – C. Wigley

Concept of Operations

The concept of operations is depicted in Figure 5.



6

Figure 5 Concept of Operations for SpaceX Dragon missions to ISS and experimental duration on ISS.

Pre-Flight / Ground Operations Concept

- Nominal passive stowage turnover at ~L-9 weeks
 - Habitats and passive stow kits
 - Animals are prepped for flight on the ground at KSC ACF
- New facility under construction
- Modular in parking lot near the SSPF
 - Starting ~L-30 days, but may vary depending on experiment specific requirements
- Late turnover at KSC
 - Currently L-25 hours
- Pending further discussions with SpaceX and KSC

- Ground Control Animals
 - Two options
 - KSC
 - Transport animals to ARC after launch
 - Use environmental chamber to simulate ascent and ISS environments
- Temperature, humidity, CO2
 - Working with SpaceX to get ascent data near real time to allow for ground control start as early as L+48 hours

On-Orbit Operations Concept

- Hatch Open
 - Crew health check of animals ASAP (visual) (repeat every 24 hours until animals transferred to Habitat on ISS)
 - Unpack Habitat and associated kits, configures Habitat for ops in EXPRESS
 - Transfer animals from Transporter to Habitat using the Access Unit
 - Transporter is bagged and returned to vehicle for return flight as passive stowage
- Daily Operations
 - Water check (visual) by the crew every 3 days
 - Once daily health checks performed via video downlink
- Science Operations
 - Timeline dependent on specific science requirements
 - Animals moved to the MSG using Access Unit for science ops
- anesthesia, euthanasia, dissections, bone density scan using densitometer, etc.
- Samples are preserved and placed in storage (conditioned or ambient as required)
 - Animals can be transferred to clean Habitat after 30 (TBR) days for extended operations. Used Habitat is bagged and returned as passive stowage on the next available SpaceX flight.

Current Science Operations Intended to be Supported

- Euthanasia via IP injection of Euthasol
- Simple dissections
 - Spleen and liver
- Tissue preservation
 - Fixation
 - Tissue transfer (solution to solution)
 - Tissue freezing
 - Carcass cool down and freezing
- Bone Densitometry

Post-flight Operations Concept

- Samples offloaded from recovery ship at one of 4 ports.
 - Return port is determined by SpaceX after undock.
- Early destow (R+ 48 hours) of conditioned stowage samples handed over to JSC cold stowage team at the dock, and transferred to nearest airport.
- Sample Return Options
 - Received by ARC at the airport (R+51 hours)
 - Received by PI at the airport (R+51 hours)
 - Flown to JSC by the Cold Stowage team and then shipped to ARC/PI using standard JSC conditioned stowage processes
- Currently no capability to support return of live animals
 - Under discussion with JSC

X. Roadmap to Multiple Generations – A. Ronca

As spaceflight offers the opportunity for unique insight into the role of forces omnipresent on Earth (but absent in orbital flight) can actively shape genomes in ways that are heritable, the 2011 Decadal Survey identified the following Research Priorities for Reproduction and Developmental Biology:

- Studies should be conducted on transmission across generations of structural and functional changes induced by exposure to space during development.
- Such research will provide vital fundamental information about how genetic and epigenetic factors interact with the environment to shape gravity dependent processes, and their penetrating influence across generations.

The Roadmap to Multiple Generations in Microgravity is depicted in Figures 6 and 7. If F0 are bred on ISS, the first totally space-adapted organisms (and their germ-cell lines) will be F2.

Importantly, achieving this goal will require that a series of steps or milestones be completed 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. Ideally, singular flights will verify that breeding, birthing and weaning occur successfully before multiple generations are attempted. At each step along the roadmap, multidisciplinary science studies will be possible and encouraged.

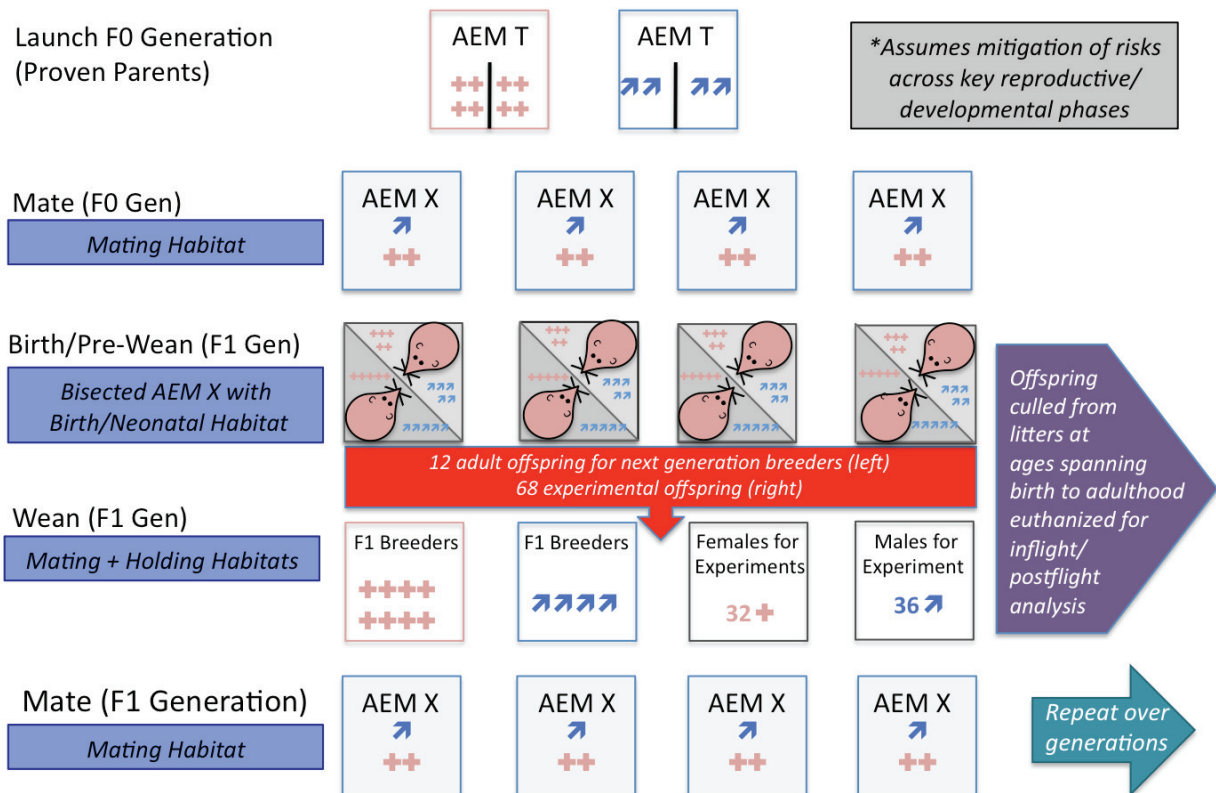


Figure 6 Roadmap to Multigenerational Generations (Rat) in Microgravity

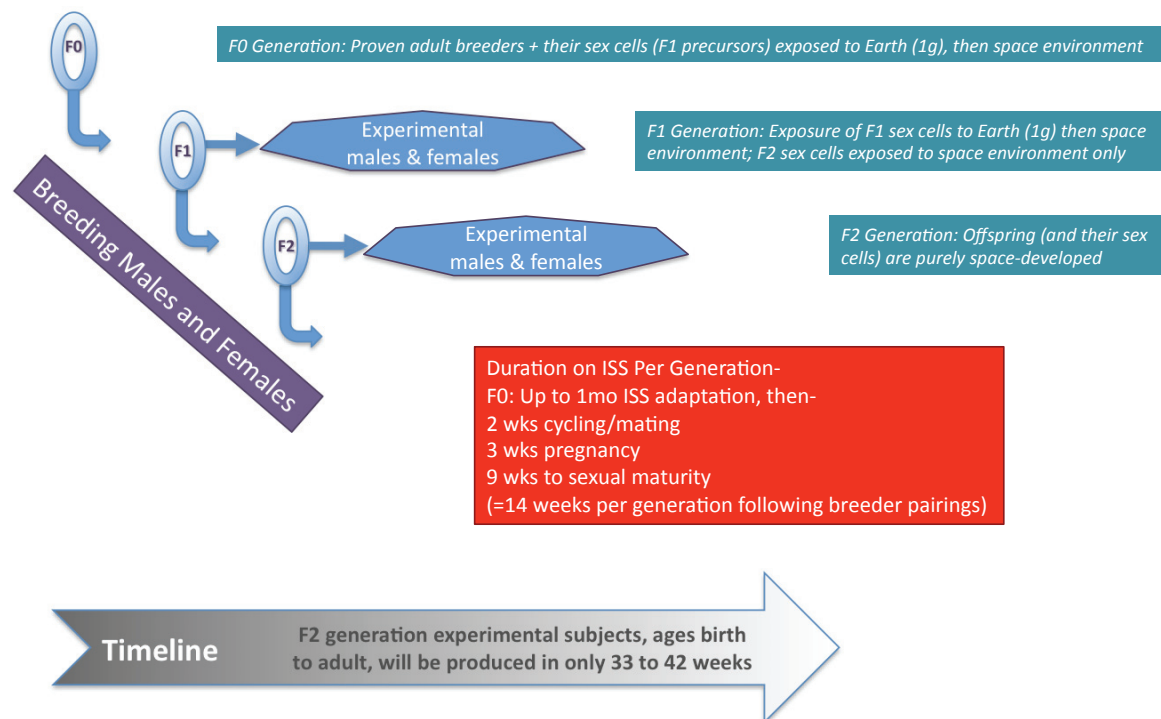


Figure 7 Multigenerational Timetable and Earth/Space Exposures per Generation.

XI. Closing

The Mark III Rodent Habitat Workshop assembled a diverse range of science, engineering and program experts to identify necessary requirements and specifications for 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) 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’ 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 knowledge gaps presented on page 23 of this report. 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.

XII. Appendix

1. Individual Presentations are available upon request

2. Additional Comments from Shawn Bengtson

The following is a summary based on my notes during my participation, discussions and inputs, during the ISS Rodent Habitat Mark III for Reproductive Biology Workshop.

Although the majority of what follows may be considered beyond the scope of the intent of the workshop from a “Level 1” perspective, in my view, the potential augmentations and inclusions are worthy of consideration and further evaluation in the early stages of habitat development.

Environmental Enrichment

As stated in the Rationale section of the Science Requirements Envelope Document (SRED)

3.1.1: “Animals shall be provided housing and resources according to their physical, psychological and behavioral needs during transport and on-site.” Consider expanding this section statement to include the “AEM ISS habitats.”

A key element for successful proof-of-concept is to provide components that address the psychological well-being of the model organisms (rats or mice) during ground and in-flight experimental operations that intend to promote the successful breeding within the Animal Enclosure Modules (AEMs). Providing components for pre-/post-bred dams to exercise species-specific instinctual behaviors exhibited such as those during courtship, copulation, pre-parturition, parturition and postpartum time points may prove to enhance the overall success. Permitting a laboratory animal (rats and mice specifically) to exhibit species-specific behaviors will result in fewer behavioral abnormalities or other pathologies (Abigail L. Smith 2005). This concept applies to nominal social behaviors and behaviors exhibited during pre-breeding courtships, breeding and post-partum performance. One must also consider importance of these provisions in order to promote a greater chance of overall receptivity success by both dam and sire in a microgravity environment.

Nestlets / Tube Shelter

It is recommended that a nest material be identified and considered for inclusion to the current AEM environments with the following considerations: Biologically inert material with nonabsorbent properties given the conditions and limited access for replacement.

The following are potential materials for consideration:

- Synthetic cotton fiber sheets
- Synthetic wool fiber sheets
- A breeding-wedge location applied/attached in the AEM with consideration for change-outs by crew (if necessary).
- A synthetic material that can remain in habitat until weaning of pups and then removed

Note: During workshop discussions on this topic, it was proposed that a synthetic material such as Polytetrafluoroethylene (PTFE) fiber sheets could be a candidate material for potential use given its non-porous and non-absorbent properties. In subsequent review through literature search of PTFE for potential rodent nesting applications, the existence of published toxicity data was found in both mouse and rat. Subsequent pathologic findings resulted in a high level micro-particulate PTFE inhalant accumulating in lungs of the animals exposed to this material. I have since made contact with several manufacturers of Nestlet/nesting product materials that support “clean room” habitat requirements. Any information obtained will be forwarded for evaluation and considerations.

Additional potential benefits for enrichment inclusions

Though the rodents are currently classified as non-Act species, including species-specific enrichment could address areas of concern by an IACUC and potentially AAALAC in using the “Guide” housing parameters for such as the available floor space within the AEMs:

“Adjustments to the amount and arrangement of space recommended in the following tables should be reviewed and approved by the IACUC and should be based on performance indices related to animal well-being and research quality... with due consideration of the AWRs and PHS Policy and other applicable regulations and standards. It is not within the scope of the Guide to discuss the housing requirements of all species used in research. For species not specifically indicated, advice should be sought from the scientific literature and from species-relevant experts.”

In other words, it is up to the IACUC to approve the overall animal space within a habitat/housing enclosure by utilizing performance standards. Including enrichment as previously described may aid the IACUC in establishing these performance standards and habitat-space considerations should this be an issue.

Development, Evaluations and Flight Candidate/Ground Control Animal Selection

It is recommended that protocols be developed to establish criteria for the selection of candidate animals for transport to ISS for intended breeding. Following are some elements to be considered:

Proven breeders

As discussed, the average success of first time breeding of rats can be as low as ~ 60%; therefore at least one successful breeding (sires and dams) prior to transport to ISS is recommended. In addition, this could be an opportunity to evaluate and record individual pre-mating/courtship behaviors of potential candidates recorded for future comparison during breeding opportunities aboard the ISS. Other areas for flight animal selection:

- Lactation/Nursing proficiency of the dam
- Proficiency of sires
- Identification of dam post-natal behaviors
- Gatherer (pups)
- Nesting behavior

Vibration and Noise Conditioning

Consideration for the development of protocols for the conditioning of flight animals to vibration and noise frequencies that will be experienced during transport to potentially mitigate negative responses/stress when exposed during launch and placement on ISS. During the animal selection process, this could be a useful element for identifying animals having a higher tolerance for launch and potential platform stressors that could affect breeding potentials. Intermittent exposure to mild low-frequency vibration and noise from post-natal periods to breeding age could prove to be beneficial.

Thermo imaging/Video Thermography

A common area of interest during ISS experiments was animal temperature regulation. It was suggested that thermo-imaging technology be reviewed for potential integration to the current AEM design for the intent of evaluation of animal surface temperatures. This capability could augment the visual veterinary evaluation and assessment of flight animals, pups and huddle dynamics, in addition to providing a parameter for adjustments to the AEM ambient temperature and airflow.

In addition, thermography is becoming increasingly used in veterinary medicine to evaluate animal surface temperatures, blood flow/perfusion and for thermoregulatory evaluations. Thermography can provide a visual management tool for diagnosing and monitoring of flight animal/pup status. Veterinary thermal imaging is passive/non-invasive, emits no radiation, can be repeated as frequently as required and could provide useful data during the stages of rodent-pup development.

Other Considerations

During the Mark III Workshop, the Animal Development Habitat developed by STAR Enterprises (Dr. Jeff Alberts) was provided for participant review. Various components of this habitat such as the nest entrance aperture, the spring-loaded rodent nest-bed along with other design features should be considered for integration into the current AEM systems. Many of the issues discussed such as pup nest-excursions and the dams ability to retrieve pups (and escape the litter at later developmental stages), isolate pup huddling and relative confinement to aid the dam in nursing of litters, could be addressed by inclusion of the Animal Development Habitat design features.