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invited review

Hindlimb unloading rodent model: technical aspects

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Morey-Holton, Emily R., and Ruth K. Globus. Hindlimb unloading rodent model: technical aspects. *J Appl Physiol* 92: 1367–1377, 2002; 10.1152/japplphysiol.00969.2001.—Since its inception at the National Aeronautics and Space Administration (NASA) Ames Research Center in the mid-1970s, many laboratories around the world have used the rat hindlimb unloading model to simulate weightlessness and to study various aspects of musculoskeletal loading. In this model, the hindlimbs of rodents are elevated to produce a 30° head-down tilt, which results in a cephalad fluid shift and avoids weightbearing by the hindquarters. Although several reviews have described scientific results obtained with this model, this is the first review to focus on the technical aspects of hindlimb unloading. This review includes a history of the technique, a brief comparison with spaceflight data, technical details, extension of the model to mice, and other important technical considerations (e.g., housing, room temperature, unloading angle, the potential need for multiple control groups, age, body weight, the use of the forelimb tissues as internal controls, and when to remove animals from experiments). This paper is intended as a reference for researchers, reviewers of manuscripts, and institutional animal care and use committees. Over 800 references, related to the hindlimb unloading model, can be accessed via the electronic version of this article.

simulated weightlessness; hindlimb suspension; altered gravity; mechanical load; animal model

GROUND-BASED MODELS PLAY A significant role in space exploration because they provide data critical for the design of spaceflight experiments. The hindlimb unloading rat model has been accepted by the scientific community as the rodent model of choice for simulating spaceflight, and its use will likely increase during the space station era. Excellent reviews of scientific results obtained from hindlimb unloading studies are available in the literature, but no publication has provided in-depth technical information useful to individuals and institutions interested in the model. The purpose of this technical review is to help investigators, institutions, and reviewers of manuscripts understand the rationale and historical aspects of the development of the technique as well as the multiple factors that may affect results obtained from hindlimb unloading model experiments. The standard operating procedure (SOP) for hindlimb unloading with applications to young and adult rats was updated and approved by the National Aeronautics and Space Administration (NASA) Ames Research Center (ARC) Institutional Animal Care and Use Committee on August 8, 2001. A copy of the SOP can be obtained by contacting the NASA ARC Animal Care Facility manager. A list of over 800 references published to date that used this model system is now available.

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RATIONALE FOR DEVELOPMENT OF GROUND-BASED MODELS

Ground-based models that simulate spaceflight are important for many reasons. Unlike spaceflight studies, experiments on Earth can be scheduled without concern for crew time, and modifications can be made as necessary during the experiment with little impact on cost. Furthermore, manipulations can be performed without the extensive precautions required for spaceflight experiments (e.g., triple containment of chemicals and specimens), and the experimental duration can be varied with multiple time points measured within a single experiment. Experiments can be repeated and extended on a routine basis. Tissues can be taken from anesthetized animals at any time during an

experiment. None of these experimental manipulations, which as scientists on Earth we take for granted, can be made with ease in space. Finally, these cost-effective ground-based experiments serve to identify biological systems most likely to be perturbed by spaceflight. Once a ground-based model is determined to suitably simulate spaceflight, it can be used to extend and predict results from spaceflight experiments. Furthermore, ground-based models avoid several complications of spaceflight, including hypergravity and vibration during launch and landing, transportation from the landing site, and the lag time between landing and harvest of tissues.

Development of models to simulate weightlessness requires several sequential steps. The process includes *1*) defining the physiological response(s) to weightlessness that the model is intended to mimic, *2*) designing and developing the system to study those aspects, *3*) collecting and analyzing data from the model, *4*) comparing the data with spaceflight studies, *5*) defining those aspects of spaceflight that the model most closely mimics, and *6*) defining limitations of the model. The primary physiological responses of vertebrates to spaceflight include fluid shifts, repositioning of certain organs, musculoskeletal unloading, and lack of stimulus to the vestibular system (33, 45).

Models for simulating several aspects of weightlessness exist for various species. Clinostats are used primarily for plants and nonmotile cells and act to inhibit sedimentation of gravity-sensing organelles (26). Head-down bed rest of humans induces a cephalad fluid shift, minimizes the hydrostatic gradient within the cardiovascular system, causes movement of abdominal organs toward the chest, and results in the loss of impact loading on the musculoskeletal system (14). Similarly, water immersion of humans causes a rapid cephalad fluid shift, unloading of the systems immersed, and loss of impact loading (12). Immobilization of nonhuman primates by chair restraint minimizes the hydrostatic gradient and prevents impact loading (13). Hindlimb unloading of rodents in a head-down position is the newest of the models for simulating weightlessness.

EVOLUTION OF THE RAT HINDLIMB UNLOADING MODEL

This section traces the history of the hindlimb unloading model, with an emphasis on technical aspects. Many laboratories were involved in defining the essential characteristics of the model. The critical components of the model are the housing unit, the device to which the rat is attached to allow mobility, the angle of unloading, and the harness system.

In the spring of 1974, Dr. Harold Sandler, chief of the Biomedical Research Division at ARC, challenged the investigators attending a space biology program review at ARC to develop a rodent model to simulate weightlessness. Although rat models for disuse existed, they required confinement of animals in small cages, limb casting, or flaccid paralysis by nerve section or surgical tenotomy. None of the techniques produced the differential muscle atrophy characteristic of spaceflight, i.e., a decrease in mass of the extensor muscles but not of other muscles associated with movement. In addition, recovery from disuse was difficult or impossible with the existing surgical models. The new model needed to complement the prevalent method for simulating spaceflight in humans, i.e., 6° head-down bed rest (25). The physiological responses to spaceflight that the rat model had to mimic included *1*) differential muscle atrophy, *2*) cephalad fluid shift, *3*) freedom to move, eat, and groom using the forelimbs, *4*) unloading of the hindlimbs without paralysis so that animals could recover from unloading, and *5*) normal weight gain in growing rats or minimal weight loss in adult animals throughout the experimental period. Data from the model would be validated using spaceflight data to define the limitations of the model. If the proposed NASA rat model yielded experimental results similar to flight data, then it would be invaluable for establishing the time course of physiological changes, studying mechanisms responsible for the observed changes, and, ultimately, predicting the effects of weightlessness.

Model testing was initiated in 1975. The first model was very simple; however, this model provided valuable lessons, leading to suggestions for modifications ultimately incorporated into succeeding designs. The first full-length paper describing the model appeared in 1979 (29). This model used a hexcelite back harness and a cantilevered rotating beam that allowed the head-down animal to move in a 360° arc. Data were compared with the weight and bone changes found in Cosmos 782 and 936. The author concluded that the changes in hindlimb-unloaded rats were similar to spaceflight. A paper from Musacchia and colleagues (31) quickly followed the first publication. Their model used a denim harness and a rotating beam that allowed the animal to move in a 140° arc. Body weight and food consumption in the hindlimb-unloaded rats were significantly less than those of the control group, and the animals exhibited adrenal hypertrophy at the end of the 7-day experiment. The authors concluded that the muscle changes were similar to those found during spaceflight and recovery from spaceflight. To determine whether the cephalad fluid shift contributed to changes in metabolism, Musacchia and colleagues (11) included a horizontal control and found that the head-down position was required for the diuresis and natriuresis that occurred during hindlimb unloading. Tipton and colleagues (41) modified the model to measure muscle changes and blood flow. Deavers et al. (11) and Bouzeghrane et al. (7) advocated for a horizontal control, particularly for studies investigating fluid shifts.

The importance of the angle of unloading was addressed by Hargens et al. (21). They found that the angle of unloading (i.e., the angle formed between the torso of the animal and the floor of the cage) determined the amount of weight supported by the forelimbs as well as the tension applied to the tail. These authors showed that the hindlimb-unloaded rat applies 50% of

its body weight to its forelimbs when the angle between the torso and the floor of the cage is 30°. As the angle increased, mechanical loading of the forelimbs declined and traction on the tail increased. If the angle was too steep, then the animals appeared stressed. A 30° angle of unloading was recommended because it provided normal weightbearing on the forelimbs, unloaded the lumbar vertebrae but not the cervical vertebrae (17), and induced a cephalad fluid shift (21).

The hindlimb unloading model has not changed conceptually from the beginning. Data from all laboratories that used the model showed differential muscle atrophy, a cephalad fluid shift, animals having the freedom to move, eat, and groom with the forelimbs, and unloading of the hindlimbs without paralysis so that animals could recover from unloading. However, the harness system and degree of mobility varied significantly between laboratories. One problem possibly related to these differences was the reduced weight gain in growing rats or weight loss in adult animals that persisted throughout the experimental period. Harnesses tested at ARC included a combination of elastic and Velcro straps and hexcelite bonded with an epoxy resin to the back of the rat (29). Each of these harnesses was only partially successful, and less stressful harness designs were sought. The concept of a tail harness originated with our Russian colleagues, who used a plaster-of-Paris mold for tail traction (23). In the early 1980s, orthopedic surgeons from the University of Southern California toured our laboratory and recommended that the tail cast be replaced with the tape that they used for placing human limbs in traction. Traction tape could be applied to an unanesthetized animal and would allow the tail to grow without restriction. In fact, the body weights of growing rats unloaded with the use of tail traction remained comparable to controls fed the same amount of food (i.e., group-mean-fed controls), in contrast to rats unloaded with the use of back harnesses (47). Tail traction appears to be less stressful to animals than whole body harnesses, as assessed by corticosterone levels and adrenal, thymus, and body weights (20, 39).

Some investigators have referred to the hindlimb unloading model as a model for disuse. Although this term may be appropriate for bone, electrical activity continues in muscles, suggesting that hindlimb unloading is not a muscle disuse model (15). Another important point about hindlimb-unloaded muscle is that muscle atrophy is not limited to postural or slow-twitch muscles because other extensor muscles also exhibit wasting during hindlimb unloading (19).

In conclusion, the use of tail traction in the hindlimb unloading model has become the technique of choice for studying spaceflightlike changes in rats and mice (Fig. 1). Although the rat hindlimb unloading model was initially developed to study musculoskeletal, cardiovascular, and metabolic changes caused by weightlessness (29), it also has been useful for investigating responses of many other physiological systems to unloading and the recovery from unloading on Earth (see list of accompanying references[1]).

COMPARISON WITH SPACEFLIGHT DATA

The development of the hindlimb unloading model coincided with cooperation between the United States and the USSR on the Cosmos spaceflight missions. These unmanned flights (which began in 1975) were 2–3 wk in duration, with studies limited to preflight and postflight observations. Data from these spaceflight experiments were compared with the hindlimb unloading model and consequently dictated the duration of the initial hindlimb unloading studies. The last rodent flight in this series, Cosmos 2044, was the first international spaceflight mission with multiple investigators and included the hindlimb unloading model as a control group coinciding with the flight (6, 9). Both spaceflight and hindlimb-unloaded animals showed similar atrophic changes in muscle and bone. Responses in heart, pulmonary, intestine, immune, endocrine, and reproductive functions were similar in both hindlimb-unloaded and spaceflight animals. In contrast, several physiological parameters, such as liver histology and metabolism, neural, red blood cell number, plasma constituents, and hypothalamic pituitary factors, differed in several respects between the two groups (6). However, hindlimb-unloaded rats were sampled 15–60 min after reloading, whereas the flight rats were euthanized 8–11 h postflight (35). The time differential might explain some of the differences between the ground-based and flight groups.

Although Cosmos 2044 was the first experiment to include a hindlimb-unloaded group as part of a spaceflight experiment, other investigators have compared data from hindlimb-unloaded experiments and spaceflight experiments, confirming that hindlimb unloading and spaceflight induce similar, although not always identical, physiological responses in many systems (e.g., see Refs. 8, 30, 42, 46).

Caution is necessary when comparing results between spaceflight and hindlimb unloading experiments. For example, the entire body is unloaded during spaceflight, whereas the forelimbs, head, and upper back remain weightbearing in hindlimb-unloaded animals. The hindlimb-unloaded group does not experience launch, landing, or transportation to and from the launch and landing pad. Fluid shifts may be greater in hindlimb-unloaded rats because the animal is a quadruped and fluid redistribution may be limited in rats during spaceflight. Despite these cautions, studies in hindlimb-unloaded rats have been instrumental in defining the time course and mechanisms of physiological changes that occur due to unloading.

TECHNICAL AND METHODOLOGICAL DETAILS

The hindlimb unloading technique has been approved by the NASA ARC Animal Care and Use Com-

[1]The reference list can be viewed at http://jap.physiology.org/cgi/content/full/92/4/1367/DC1.

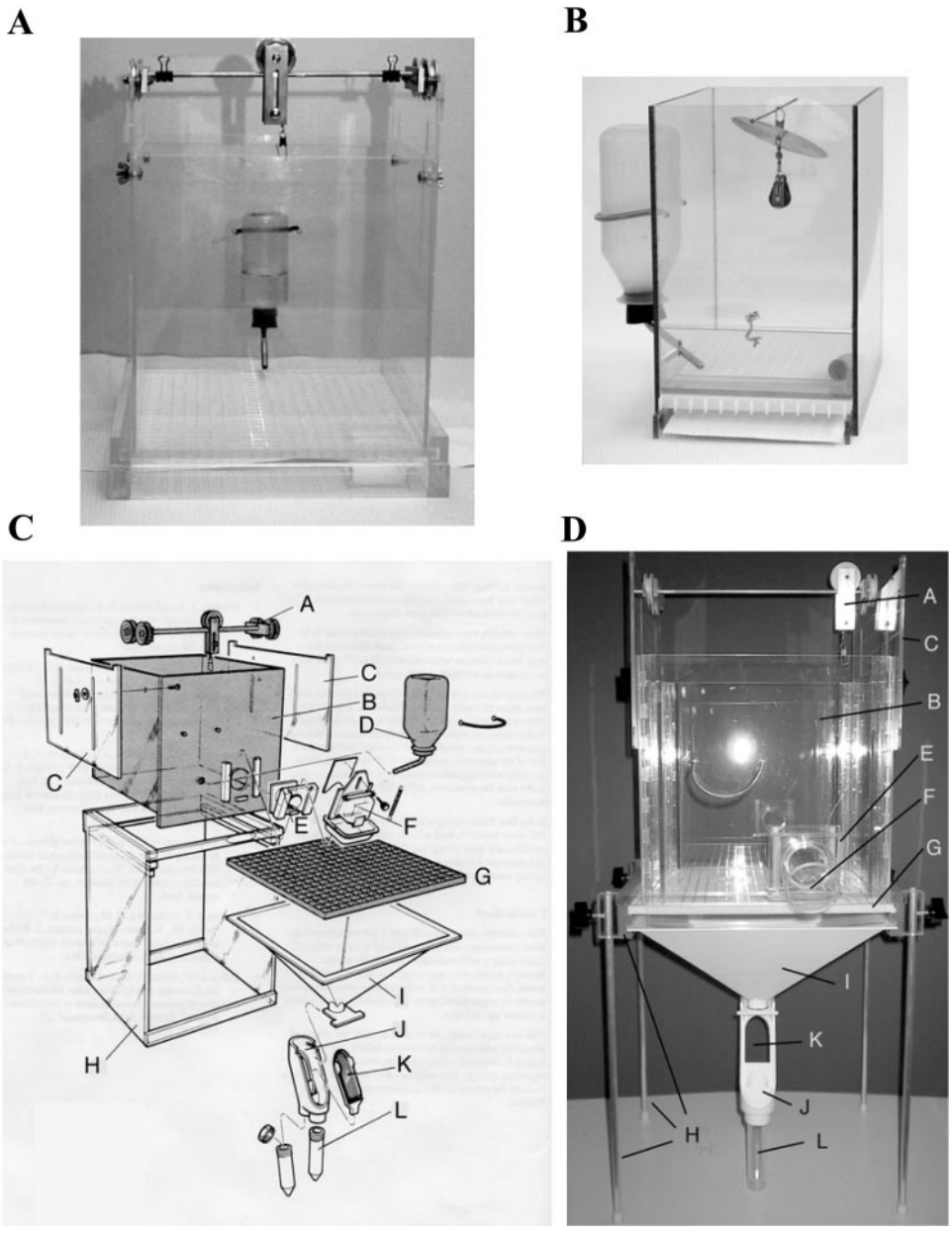

Fig. 1. Depiction of several cages used for hindlimb unloading experiments. *A*: rat cage described by Wronski and Morey-Holton (47). The cage is ~12 in. wide × 12.5 in. deep × 12 in. tall. The side extensions (C in *panel C*) can increase the height to 18.5 in. The unloading device allows free movement throughout the cage. *B*: mouse cage designed by Dr. Marjolein van der Meulen (Cornell University). The cage dimensions are ~7.6 × 11 × 12 in. Movement of the unloading device in this cage is along a single axis, although the animal retains access to the entire cage. The hook latches a removable food dish in the bottom of the cage. The round metal disk above the unloading device prevents the mouse from climbing up the unloading apparatus. *C*: detailed version of the metabolic cage developed by Arnaud and collaborators (22). *D*: cage designed by Dr. Russell Turner and colleagues at Mayo Clinic. The Mayo cage has incorporated additional features, including cage tops that fold for storage, the ability to replace the collection funnel with a tray for bedding, and lowering the entire unit for studies that do not require metabolic collections. This design is a hybrid of cages shown in *A* and *C*. In *C* and *D*, A = unloading device, B = cage top, C = height extender (the wheels on the unloading device roll along the top of the height extender or the sides of the cage), D = water bottle assembly, E = tunnel, F = food cup, G = cage floor, H = cage base, I = collection funnel, J = separator housing, K = linear diffuser, and L = collection tube.

mittee as a rat model for simulating spaceflight. The approval was based on data showing that indicators of stress (i.e., thymus weight, adrenal weight, corticosterone levels, food consumption, body weight) in hindlimb-unloaded animals did not markedly differ from those in control animals. Food consumption may decrease for the first few days of the experiment, but the animals adapt to the system quickly, as indicated by essentially normal food consumption and stable or increasing body weight throughout the experiment.

The following paragraphs provide detailed information on required and optional supplies, preparation of the animal, and the procedure as performed at NASA ARC.

Supplies. Required supplies for hindlimb unloading include *1*) a gauze or towel soaked with 70% alcohol or alcohol wipes, *2*) tincture of benzoin aerosol spray (Professional Packaging, Aurora, IL), *3*) a towel for restraining the animal, *4*) traction tape or similar adhesive traction strips (e.g., Skin-trac, 3 × 40 in.; Zimmer, Warsaw, IN), *5*) a plastic tab with attached paperclip loop (the dimensions of the tab are sized to the weight of the rat) (Fig. 2), *6*) filament tape (0.75-in.-wide roll can be purchased at any stationery store), *7*) gauze bandage for wrapping the tail, *8*) a hindlimb unloading cage, and *9*) plastic-backed absorbent paper or shavings that fit under the cage to absorb urine if appropriate.

Optional supplies include *1*) a hair dryer, *2*) Bitter Apple spray, Chew Guard, or equivalent for use with adult rats (obtained from a veterinary supply company), *3*) wooden dowels (can be purchased at any hardware store) or nylon chew bars (can be purchased at any pet shop), and *4*) a pen for marking the rat's tail.

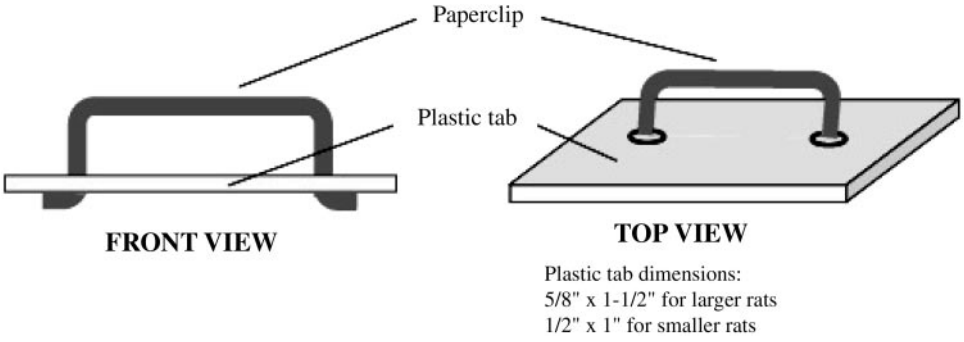

Fig. 2. To make a plastic tab, a paperclip is inserted through small holes drilled in a piece of plastic, bent along the underside, and trimmed with wire cutters.

Preparation. Strips of the traction tape and filament tape are prepared before the experiment. The width of strips of traction tape depends on the width of the rat's tail. The strips are narrow enough so that they do not touch when placed on opposing sides of the tail. The plastic tab should be held at the base of the tail just above the hair line, and the length of tape required should be determined as that which extends about two-thirds the length of the tail on two sides of the tail (see Fig. 4). For example, we cut strips to ~12 in. long and ~0.25 in. wide for young animals (~150 g). The paper protecting the traction tape is cut in the middle of the strip and peeled back (but not removed) ~0.5 in. on each side. The tape is threaded through the paperclip loop on the plastic tab until an equal amount of tape is on either side and then pressed to the plastic tab so that it adheres (Fig. 3).

Handling of the animals begins ~1 wk before the procedure. The animals are acclimated to their cages at least 2 days before beginning the hindlimb unloading. If the animals are shipped by air freight, then a longer acclimation period is advisable.

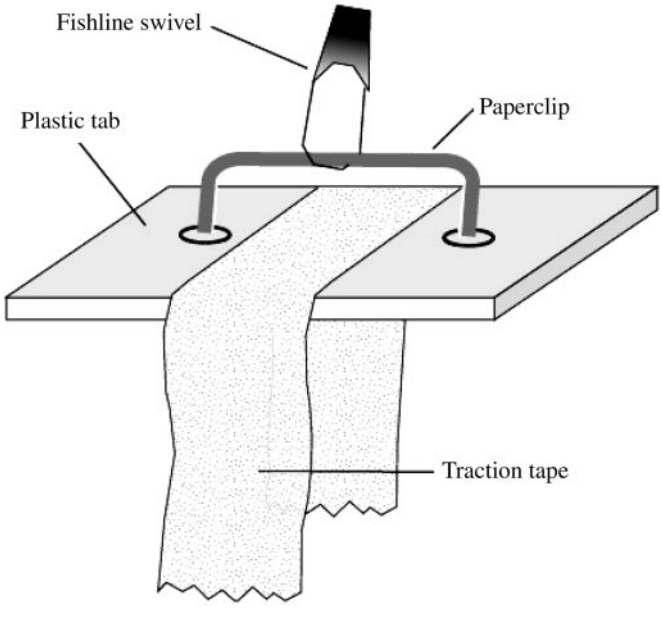

Fig. 3. Traction tape is threaded through the paperclip loop and adhered to the tab.

Procedure. On the day of hindlimb unloading, the rat is wrapped loosely in a towel (the rat runs into the towel and away from the operator). A second person can assist in keeping the rat in the towel, although an operator familiar with the procedure usually can perform the procedure alone. We use minimal restraint to avoid stressing the rat during the procedure. We do not anesthetize animals and recommend that anesthesia be avoided if possible because recovery from anesthesia may compromise the animals' ability to readily adapt to hindlimb unloading. If anesthesia is used, we recommend at least a 24-h recovery period after anesthesia and before hindlimb unloading.

We clean the tail with alcohol, removing all the dead or dirty skin. The tail is allowed to dry, which usually takes 1 min or less. The tail is then sprayed with a generous amount of adhesive spray (e.g., tincture of benzoin) and allowed to dry for 1–5 min. The rat can be placed in its cage while the adhesive spray is drying or a hair dryer can be used on a low heat setting to accelerate the drying process. When the spray is tacky, a strip of traction tape, preattached to the plastic tab (Fig. 3), is attached to the tail. Traction tape is cut to the correct length for the rat. The paper backing is removed from the tape, and the tape is attached to the tail beginning at the base of the tail just above the hair line. The traction tape is gently pressed to the tail so that it sticks along the tail's surface. The traction tape is narrow enough so that the tape on one side of the tail does not come into contact with the tape on the other side of the tail (Fig. 4). Traction tape is secured to the tail with two strips of filament tape (~0.25 in. wide × 1.5 in. long). One strip of filament tape is placed around the base of the tail over the ends of the traction tape, and a second strip is added about half-way up the tape (Fig. 4). The filament tape must be loose enough to allow normal blood circulation but tight enough so that the traction tape will not peel away from the tail. Gauze bandage can be wrapped around the tail to protect the traction tape and to deter the rat from peeling off the tape. Because the tail is critical for thermoregulation in the rat, only the area covered with traction tape should be wrapped with gauze bandage, i.e., primarily around the base of the tail. A line can be drawn with a marking pen at the bottom of the traction tape to monitor movement of the tape. The rat is now ready to be attached to the fish-line swivel that hangs

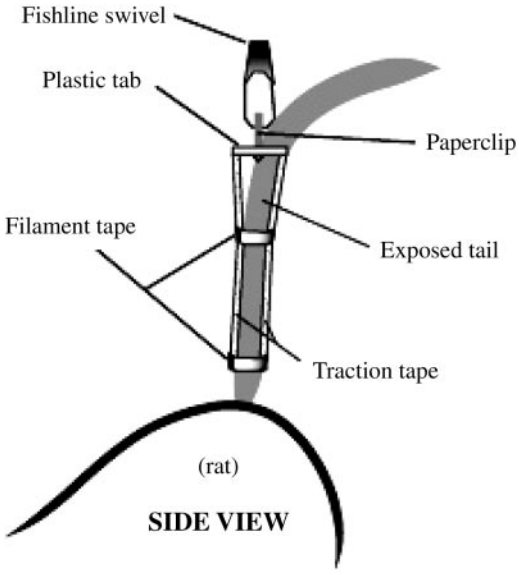

Fig. 4. A side view of the traction tape and filament tape attached to the tail.

from the unloading device on top of the hindlimb unloading cage (Fig. 1*C*).

If the rat is allowed to move on an *x*-*y* axis and rotate 360° so that it has access to all areas of the cage, then it appears to adapt more readily. Our unloading device consists of two sets of bearings connected by a metal rod and a fifth bearing that rolls along the metal rod; a fish-line swivel is connected to the fifth bearing (Fig. 1*A*). The unloading device, located at the top of the cage, rides along two parallel sides of the cage. The pulley system and swivel (Fig. 1*C*) are designed to impart minimal resistance so that the animal can move easily and without effort using its front paws. The body of the rat makes about a 30° angle from the cage floor, and thus the back feet of the rat do not touch the grid floor. The hind legs should be manually extended to assure that the toenails do not touch the floor. The angle and height of the rats are checked, and adjusted if necessary, on a daily basis. The cage sides are adjustable to increase or decrease the height of the unloading device. The animal must be able to reach food and water after the adjustments are made.

To prevent animals from pushing against the sides of the cages with their hindlimbs, large binder clips can be placed atop the cage sides to restrict movement of the unloading device. Several papers describe various cage designs, including metabolic cages for collecting urine and feces (22, 29, 34, 47).

Absorbent paper is placed under the cages to absorb urine unless a metabolic cage is used. The papers are changed as needed but not less than twice per week. Normal bedding material placed in a tray under the cage can also be used for this purpose.

Control animals should be kept in identical cages if possible. An unloading device is not needed for the control cage, although a cover for the top of the cage is necessary to ensure that the rat does not escape. Extra cage floors make excellent cage tops as they permit air circulation. A weight placed on the cage top may be necessary to keep larger animals from escaping.

A wooden dowel or nylon chew bar (e.g., Nylabones) is provided to each rat for chewing. A piece of dowel should be ~1.5 in. long and ~0.75–1 in. diameter. This item reduces the likelihood that the rat will chew through the floor of the cage.

The cage should be disassembled for cleaning. In the preliminary bath, each part is washed with hot water and if necessary scrubbed to remove any visible organic matter. The parts are then completely submerged in a disinfectant solution for at least 0.5 h for plastic pieces and a shorter time for the metal pieces. The metal pieces should be dried when removed. The plastic pieces for the cages are rinsed, reassembled, and allowed to air dry.

If adult male Sprague-Dawley or Wistar rats (~400 g) are used, then the following precautions are recommended. The rats should be handled several times a day for at least 1 wk before the experiment begins. Traction tape should be placed at the base of the tail (unlike the younger animals, the older animals appear to adapt to traction much better when the traction tape begins at the very base of the tail). The adhesive spray appears to be less well tolerated by adult animals and any tincture sprayed on the hair should be removed. The animal must be held until the spray dries so that the spray is not spread. The spray contains alcohol, which is not well tolerated if the animal has any abrasions. The base of the tail can be coated with Bitter Apple or Chew Guard or equivalent to discourage the animal from chewing on the traction tape. The animals must be removed from the cage for this procedure because the rat will avoid any cage area or food contaminated with this substance. Any tail with abrasions should be treated immediately with antibiotic cream and zinc oxide; only the abrasion and no other part of the tail should be coated with these substances, i.e., a minimal amount of compound should be used.

Animals should be inspected daily by the attending veterinarian and/or registered veterinary technicians to assure the health of the animals. Specific points of inspection include the following: *1*) overall appearance and activity (during the initial days of hindlimb unloading, porphyrin may appear around the nose and eyes in varying degrees, although this typically disappears or starts to disappear by the third day), *2*) evidence that the animals are eating, drinking, and able to move freely, and *3*) inspection of the tail. Typically, the ends of the tails in hindlimb-unloaded rats are only slightly engorged and appear pink. If the end of the tail appears swollen or has a blue tint, corrective actions need to be taken immediately to ensure proper circulation in the tail. Male rats are checked daily for sperm plugs in the urethra. Sperm plugs are expressed manually if present. In addition, animals should be inspected carefully twice per day by the investigator or his/her staff to determine whether any experimental animal is slipping out of the harness. If the tape on the tail is too loose, then the end of the traction tape will slip away from the ink mark at the base of the tail.

When this happens, filament tape should be replaced and tightened slightly. After the filament tape has been in place for 2–3 days, it should be removed or cut in two or replaced with looser tape to avoid restriction of tail growth or circulation.

EXTENSION OF THE SYSTEM TO MICE

The hindlimb unloading model has been adapted for use on mice to study the metabolic and immune responses of mice to antiorthostasis (3, 4, 8, 40), as well as the musculoskeletal responses to unloading (36–38). With the increasing availability of transgenic and mutant strains of mice, the hindlimb unloading model is now being used to unravel molecular mechanisms that mediate responses to hindlimb unloading and antiorthostasis (10, 24, 28, 44). With respect to the skeletal system, there is a strong genetic component to peak bone density. Recent evidence shows that the skeletal responsiveness to unloading and exercise is partially dependent on the specific mouse strain (1, 2, 27, 43). Therefore, it is important to test the sensitivity of the appropriate wild-type strain of mice to hindlimb unloading for each mutant or transgenic line under study.

Whether hindlimb unloading causes a systemic stress response in mice is less well-resolved than for rats. In some studies (8, 36–38), hindlimb unloading was shown to cause an increase in glucocorticoid levels and adrenal mass of mice and/or a decrease in body mass relative to controls fed ad libitum. Furthermore, Simske and colleagues (36–38) report that hindlimb unloading causes osteopenia in bones from the forelimbs as well as hindlimbs. However, in our hands, hindlimb unloading for 4 wk does not always cause a reduction in bone mass of the forelimbs relative to group-mean-fed controls, nor does it reduce body mass relative to controls (M. van der Meulen and R. Globus, unpublished observations). The discrepancies in results obtained between studies performed in different laboratories may be due to differences in unloading technique (e.g., angle of unloading), feeding regimen of control groups, and/or specific mouse strain.

A number of adjustments to the hindlimb unloading system are needed to accommodate the smaller body size and differences in behavior of mice compared with rats. Such differences include the use of smaller cages (Fig. 1*B*), inclusion of a device to prevent mice from climbing the unloading apparatus, and holding mice by the scruff of the neck (rather than by towel restraint) when traction tape is applied to the tail.

IMPORTANT ENVIRONMENTAL PARAMETERS

When designing habitats for unmanned spacecraft, the biologist must supply the engineer with information about nutrient intake, metabolic output, and environmental requirements for that species. Such data are critical for the design of optimal animal habitats. Because of our experience with designing habitats for spaceflight, we appreciate the importance of an optimal environment for ground-based experiments as well as space-based experiments. The next two sections elucidate important environmental and physiological parameters for hindlimb unloading experiments. Our attention to the animal's environment and husbandry may be the reason that endocrine stress is minimized in our experiments (17, 20).

Housing. Our experimental animals are housed in a separate room, and only personnel associated with the experiment are allowed in that room during the experiment. Our controls are housed in cages that are identical to those used for hindlimb unloading, except that the unloading device (Fig. 1*C*) is removed and replaced with a top cover to keep the control animal from escaping.

Room temperature. We find that maintaining the room temperature between 24.5 and 25.5°C is important for animals housed individually. In contrast, National Institutes of Health guidelines for rodents specify a room temperature of 18–26°C for mice and rats in standard, group-housed cages (*Guide for the Care and Use of Laboratory Animals*, Washington, DC: National Acad. Press, 1996, p. 28–32). The temperature we use is approximately the lower range for the thermoneutral zone (TNZ) for rats and mice (i.e., 26–34°C) (18). TNZ is defined as the temperature range in which the metabolic rate is minimal and theoretically equal to the basal metabolic rate. As the ambient temperature is reduced below the TNZ, heat production must increase to maintain a normal body temperature. Individually housed animals are more likely to maintain body weight at this higher room temperature. Hypophysectomized animals require a room temperature between 26 and 28°C to adjust for loss of the pituitary function, which compromises body temperature regulation.

IMPORTANT PHYSIOLOGICAL CONSIDERATIONS

Body weight. Differences in body weights between hindlimb-unloaded and normally loaded control groups can influence interpretation of experimental results. Body weight is an important and easily accessible parameter that reflects the health of a rat. Thus we strongly recommend that body weight data be provided with submitted manuscripts and that reviewers of such manuscripts insist on the inclusion of that information. If an animal loses 10% of its body weight in a 24-h period, then it may have health problems, no food, or an air bubble in the water bottle. The 10% limit or $\geq$2 SD from the mean is recommended as a limit for acceptable weight loss. Animals exceeding this limit should be eliminated from the experiment if they do not regain weight within 2 days. If experimental animals are significantly lighter than controls, then data interpretation may become difficult because differences could be due to altered body weight rather than to unloading.

The rats should be weighed daily or at least twice per week throughout the experiment. We use a tripod (Fig. 5) or a ring stand fitted with a crossbar and hook to avoid weightbearing of hindlimb-unloaded rodents during an experiment. Either device should fit onto a

Fig. 5. A tripod used for weighing hindlimb unloaded rats. We use a string of paper clips attached to the hook to adjust for height. The forelimbs of the rat should touch the balance.

balance. The rat is gently removed from the unloading device and attached to the hook so that the animal's forelimbs touch the balance while the hindlimbs remain unloaded. Weighing should not take more than 1 min. We believe it is critical that animals do not become weightbearing when they are weighed.

Age. Young rats appear to adapt more readily to hindlimb unloading than adult animals. Rapidly growing rats (<200 g) should not lose weight, although they may gain weight more slowly than controls. Adult rats (>200 g) may lose weight or gain weight more slowly for several days after initial unloading, followed by stabilization of body weights. Older adult rats (>400 g) may have difficulty adapting to hindlimb unloading, as assessed by body weight and health checks; if adult males are used, smaller strains, e.g., Fischer 344 rats, should be considered.

When designing experiments, it is important to distinguish between growing and adult rodents. Most rodents mature sexually several months before growth slows and the animals become adults, i.e., when growth curves are asymptotic. Changes in growing animals should not be directly extrapolated to adults. The growth processes of bone slow when the animal is 6–8 mo of age. Hindlimb unloading may suppress physiological processes responsible for growth but not those processes associated with the maintenance of adult tissues.

Forelimbs as an internal control. As long as the hindlimb-unloaded rat supports 50% of its body weight on the forelimbs and body weight does not differ significantly from controls, then the forelimbs can be used as effective internal controls for experiments (17, 20). If changes occur in forelimb tissues while these conditions are met, then the alterations may be indicative of systemic, rather than local, responses to hindlimb unloading. Systemic changes may include an increase in the levels of glucocorticoids or other endocrine changes. If appropriate for the system under study, then responses in the forelimbs as well as the hindlimbs of animals to hindlimb unloading should be analyzed and the results compared with weightbearing controls.

Control groups. More than one control group may be needed to correctly interpret results obtained from hindlimb unloading experiments. An experiment with insufficient control groups will have to be repeated and will use more animals than a protocol requesting adequate control groups. We have included various groups to control for body mass or experimental treatments.

Control groups for body mass have included weightbearing ad libitum, group-mean-fed, and weight-matched animals. Rats fed ad libitum weigh more than hindlimb-unloaded rats, regardless of age; the weight difference can range from ~5% in very young rats to ~20% in rats over 3 mo of age. Although weight-matched rats may be useful in some studies, the reduced caloric intake may change the behavior and physiology of this control group. To minimize weight difference in our experimental groups, we normally feed our control group the average amount of food consumed by the hindlimb-unloaded group (i.e., group-mean-fed animals) so that both groups have the same caloric intake. In our experiments, hindlimb unloading does not cause rats to stop eating, although food consumption for the first 1–2 days of unloading can drop by as much as 50%.

Another control group important for growing animals is a baseline control. This group provides tissues harvested at the beginning of the hindlimb unloading period. Comparison of a baseline group with growing, hindlimb-unloaded animals at the end of an experiment allows one to distinguish between a true tissue loss (i.e., atrophy) vs. an inhibition of growth.

When a pharmacological agent or nutritional supplement is used during hindlimb unloading, a control group that receives the same agent is essential to correctly interpret the results. In addition, the parameter of interest measured in hindlimb tissues can also be measured in tissues from the weightbearing forelimbs. Papers have been published stating that specific drugs inhibit the changes caused by hindlimb unloading. This statement is valid only if the investigators have demonstrated that the treatment alone does not change baseline values. If the drug alters the parameter measured in weightbearing control animals, then the use of untreated weightbearing animals as the sole control group limits the conclusions that can be drawn from that experiment. For example, we have shown that supplementation of the diet with calcium in-

creases bone mass in young, growing rats relative to animals fed standard lab chow (16). However, the unloaded bone mass is lower than the bone mass of weightbearing controls fed the same calcium-supplemented diet. We find a similar phenomenon when we treat growing rats with a bisphosphonate (5). The bisphosphonate increases bone mass in both weightbearing and hindlimb unloaded groups. By including a drug-treated, weightbearing control group, we are able to conclude that neither dietary calcium supplementation nor bisphosphonate treatment prevents the decrement in bone mass caused by unloading. We believe it is incorrect to conclude that a specific treatment inhibits the changes caused by musculoskeletal unloading unless the investigators have shown that the treatment alone does not affect baseline values.

WHEN TO REMOVE AN ANIMAL FROM AN EXPERIMENT

The investigator and the attending veterinarian must agree before the study begins as to the criteria for removing a rodent from the study. We use various criteria to determine when to remove animals from an experiment. One reason for removing an animal is stress. We use several parameters to assess stress during an experiment, including porphyrin around the eyes and nose and weight loss or failure to gain weight (if the animal is growing). In many experiments, particularly with older rats, we notice "red tears" or porphyrin that usually disappear within the first few days of an experiment. If porphyrin continues, then a veterinarian should evaluate the health of the rat. If a rat fails to maintain or gain weight compared with the rest of the experimental group, then that animal should be considered for removal if the weight is $\geq$2 SD from the mean. Another indicator of health that other investigators may use as a criteria for removing animals from experiments is colonic temperature.

We also remove animals that repeatedly free themselves. We check the animals twice per day during the week and once per day on weekends for the duration of an experiment. Our animal care staff also examines animals daily. Occasionally, unloading devices (Fig. 1) become stuck or fall off the track. Whether the animal is removed after this episode depends on how the animal responds over the next 2 days and its body weight compared with the rest of the hindlimb-unloaded group.

STRESS AND HINDLIMB UNLOADING

Postmortem indicators for stress include atrophy of the thymus, adrenal hypertrophy, and increased corticosterone levels. In young animals, we find thymus weight to be the simplest method for assessing stress. We do not consistently observe decreased thymus weights in hindlimb-unloaded animals compared with group-mean-fed controls. An investigator at ARC routinely measures adrenal weights in his hindlimb unloading rat experiments and has never observed adrenal hypertrophy (R. Grindeland, personal communications). However, another laboratory (11) reports adrenal hypertropy at the end of a 7-day experiment.

Plasma corticosterone levels, measured by two ARC investigators, doubled the first day of hindlimb unloading and were at ambulatory control levels by *day 2* (R. Grindeland and E. Morey-Holton, unpublished observations). Corticosterone levels in 24-h urine collections were increased only during the first 24 h of hindlimb unloading (32). We did not find changes in the level or diurnal pattern of plasma corticosterone during the fourth day of hindlimb unloading (20). In contrast, another research group (39) reported differences in plasma corticosterone on *days 1* and *3* of the experiment, with normal values achieved by *day 7*. Perhaps our animals are less stressed because of our control of the environmental and physiological parameters defined above and our use of tail traction rather than a whole body harness.

CONCLUSIONS

In conclusion, selection of the appropriate control group(s) emerges as a critical component for the design of hindlimb unloading experiments. More than one control group may be needed to correctly interpret the experimental data. We recommend analyzing forelimb tissues as well as adrenal and thymus weights to identify possible systemic responses to hindlimb unloading together with a close monitoring of body weight changes throughout the experiment. The forelimb analyses are particularly important for experiments with mice, which have not been as well studied as rats. These data are essential if the objective of transgenic and mutant mice experiments is to demonstrate that a specific gene product mediates the responses to mechanical unloading per se, rather than to systemic stress.

Environmental and physiological parameters, including room temperature, angle of unloading, and body weight, should be monitored daily. Animal housing, handling, and age are also important factors to consider in designing hindlimb unloading experiments.

The hindlimb unloading model was developed to mimic spaceflight. The model has also proven to be useful for investigating the physiological responses of both rats and mice to the recovery process associated with reloading as well as unloading. Thus the model has provided important insights into both spaceflight-based and Earth-based health problems.

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REFERENCES

1. **Akhter MP, Cullen DM, Pedersen EA, Kimmel DB, and Recker RR.** Bone response to in vivo mechanical loading in two breeds of mice. *Calcif Tissue Int* 63: 442–449, 1998.
2. **Akhter MP, Iwaniec UT, Covey MA, Cullen DM, Kimmel DB, and Recker RR.** Genetic variations in bone density, histomorphometry, and strength in mice. *Calcif Tissue Int* 67: 337–344, 2000.
3. **Armstrong JW, Kirby-Dobbels K, and Chapes SK.** The effects of rM-CSF and rIL-6 therapy on immunosuppressed antiorthostatically suspended mice. *J Appl Physiol* 78: 968–975, 1995.
4. **Armstrong JW, Nelson KA, Simske SJ, Luttges MW, Iandolo JJ, and Chapes SK.** Skeletal unloading causes organ-specific changes in immune cell responses. *J Appl Physiol* 75: 2734–2739, 1993.
5. **Bikle DD, Morey-Holton ER, Doty SB, Currier PA, Tanner SJ, and Halloran BP.** Alendronate increases skeletal mass of growing rats during unloading by inhibiting resorption of calcified cartilage. *J Bone Miner Res* 9: 1777–1787, 1994.
6. **Booth FW and Grindeland RE.** Comparison of the physiology of the spaceflight and hindlimb suspended rat. In: *Final Reports of the U.S. Experiments Flown on the Soviet Biosatellite Cosmos 2044, NASA TM 108802*, edited by Connolly JP, Grindeland RE, and Ballard RW. Moffett Field, CA: NASA Ames Research Center, 1994, p. 275–281.
7. **Bouzeghrane F, Fagette S, Somody L, Allevard AM, Gharib C, and Gauquelin G.** Restraint vs. hindlimb suspension on fluid and electrolyte balance in rats. *J Appl Physiol* 80: 1993–2001, 1996.
8. **Chapes SK, Mastro AM, Sonnenfeld G, and Berry WD.** Antiorthostatic suspension as a model for the effects of spaceflight on the immune system. *J Leukoc Biol* 54: 227–235, 1993.
9. **Cosmos 2044 mission.** *J Appl Physiol* 73: 1S–200S, 1992.
10. **Criswell DS, Booth FW, DeMayo F, Schwartz RJ, Gordon SE, and Fiorotto ML.** Overexpression of IGF-I in skeletal muscle of transgenic mice does not prevent unloading-induced atrophy. *Am J Physiol Endocrinol Metab* 275: E373–E379, 1998.
11. **Deavers DR, Musacchia XJ, and Meininger GA.** Model for antiorthostatic hypokinesia: head-down tilt effects on water and salt excretion. *J Appl Physiol* 49: 576–582, 1980.
12. **Epstein M.** Renal, endocrine, and hemodynamic effects of water immersion in humans. In: *Handbook of Physiology. Environmental Physiology*. Bethesda, MD: Am. Physiol. Soc., 1996, Sect. 4, vol. II, chapt. 37, p. 845–853.
13. **Florence G, Riondet L, Malecki H, Blanquie JP, Martin F, Viso M, and Milhaud CL.** A restraining system for rhesus monkeys used in space research. *J Med Primatol* 24: 61–67, 1995.
14. **Fortney SM, Schneider VS, and Greenleaf JE.** The physiology of bed rest. In: *Handbook of Physiology. Environmental Physiology*. Bethesda, MD: Am. Physiol. Soc., 1996, sect. 4, vol. II, chapt. 39, p. 889–939.
15. **Fournier M, Roy RR, Perham H, Simard CP, and Edgerton VR.** Is limb immobilization a model of muscle disuse? *Exp Neurol* 80: 147–156, 1983.
16. **Globus RK, Bikle DD, Halloran B, and Morey-Holton E.** Skeletal response to dietary calcium in a rat model simulating weightlessness. *J Bone Miner Res* 1: 191–197, 1986.
17. **Globus RK, Bikle DD, and Morey-Holton E.** The temporal response of bone to unloading. *Endocrinology* 118: 733–742, 1986.
18. **Gordon CJ.** *Temperature Regulation in Laboratory Rodents*. Cambridge, UK: Cambridge Univ. Press, 1993, p. 62–66.
19. **Grindeland RE, Roy RR, Edgerton VR, Grossman EJ, Mukku VR, Jiang B, Pierotti DJ, and Rudolph I.** Interactive effects of growth hormone and exercise on muscle mass in suspended rats. *Am J Physiol Regulatory Integrative Comp Physiol* 267: R316–R322, 1994.
20. **Halloran BP, Bikle DD, Cone CM, and Morey-Holton E.** Glucocorticoids and inhibition of bone formation induced by skeletal unloading. *Am J Physiol Endocrinol Metab* 255: E875–E879, 1988.
21. **Hargens AR, Steskal J, Johansson C, and Tipton CM.** Tissue fluid shift, forelimb loading, and tail tension in tail-suspended rats. *Physiologist* 27: S37–S38, 1984.
22. **Harper JS, Mulenburg GM, Evans J, Navidi M, Wolinsky I, and Arnaud SB.** Metabolic cages for a space flight model in the rat. *Lab Anim Sci* 44: 645–647, 1994.
23. **Il'in EA and Novikov VE.** Stand for modelling the physiological effects of weightlessness in laboratory experiments with rats. *Kosm Biol Aviakosm Med* 14: 79–80, 1980.
24. **Ishijima M, Rittling SR, Yamashita T, Tsuji K, Kurosawa H, Nifuji A, Denhardt DT, and Noda M.** Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin. *J Exp Med* 193: 399–404, 2001.
25. **Kakurin LI, Lobachik VI, Mikhailov VM, and Senkevich YA.** Antiorthostatic hypokinesia as a method of weightlessness simulation. *Aviat Space Environ Med* 47: 1083–1086, 1976.
26. **Klaus DM.** Clinostats and bioreactors. *Gravitational Space Biol Bull* 14: 55–64, 2001.
27. **Kodama Y, Dimai HP, Wergedal J, Sheng M, Malpe R, Kutilek S, Beamer W, Donahue LR, Rosen C, Baylink DJ, Farley J, Akhter MP, Cullen DM, Pedersen EA, Kimmel DB, and Recker RR.** Cortical tibial bone volume in two strains of mice: effects of sciatic neurectomy and genetic regulation of bone response to mechanical loading. *Bone* 25: 183–190, 1999.
28. **McCarthy JJ, Fox AM, Tsika GL, Gao L, and Tsika RW.** β-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Am J Physiol Regulatory Integrative Comp Physiol* 272: R1552–R1561, 1997.
29. **Morey ER.** Spaceflight and bone turnover: correlation with a new rat model of weightlessness. *Bioscience* 29: 168–172, 1979.
30. **Morey-Holton ER and Globus RK.** Hindlimb unloading of growing rats: a model for predicting skeletal changes during space flight. *Bone* 22: 83S–88S, 1998.
31. **Musacchia XJ, Deavers DR, Meininger GA, and Davis TP.** A model for hypokinesia: effects on muscle atrophy in the rat. *J Appl Physiol* 48: 479–486, 1980.
32. **Ortiz RM, Wang TJ, and Wade CE.** Influence of centrifugation and hindlimb suspension on testosterone and corticosterone excretion in rats. *Aviat Space Environ Med* 70: 499–504, 1999.
33. **Pace N.** Weightlessness: a matter of gravity. *N Engl J Med* 297: 32–37, 1977.
34. **Park E and Schultz E.** A simple hindlimb suspension apparatus. *Aviat Space Environ Med* 64: 401–404, 1993.
35. **Riley DA, Ellis S, Giometti CS, Hoh JF, Ilyina-Kakueva EI, Oganov VS, Slocum GR, Bain JL, and Sedlak FR.** Muscle sarcomere lesions and thrombosis after spaceflight and suspension unloading. *J Appl Physiol* 73: 33S–43S, 1992.
36. **Simske SJ, Broz JJ, Fleet ML, Schmeister TA, Gayles EC, and Luttges MW.** Contribution of dietary and loading changes to the effects of suspension on mouse femora. *J Exp Zool* 269: 277–285, 1994.
37. **Simske SJ, Guerra KM, Greenberg AR, and Luttges MW.** The physical and mechanical effects of suspension-induced osteopenia on mouse long bones. *J Biomech* 25: 489–499, 1992.
38. **Simske SJ, Luttges MW, and Wachtel H.** Age dependent development of osteopenia in the long bones of tail-suspended mice. *Biomed Sci Instrum* 26: 87–94, 1990.
39. **Steffen JM and Musacchia XJ.** Disuse atrophy, plasma corticosterone, and muscle glucocorticoid receptor levels. *Aviat Space Environ Med* 58: 996–1000, 1987.
40. **Steffen JM, Robb R, Dombrowski MJ, Musacchia XJ, Mandel AD, and Sonnenfeld G.** A suspension model for hypokinetic/hypodynamic and antiorthostatic responses in the mouse. *Aviat Space Environ Med* 55: 612–616, 1984.
41. **Stump CS, Overton JM, and Tipton CM.** Influence of single hindlimb support during simulated weightlessness in the rat. *J Appl Physiol* 68: 627–634, 1990.

42. **Tischler ME, Henriksen EJ, Munoz KA, Stump CS, Woodman CR, and Kirby CR.** Spaceflight on STS-48 and earth-based unweighting produce similar effects on skeletal muscle of young rats. *J Appl Physiol* 74: 2161–2165, 1993.
43. **Turner CH, Hsieh YF, Muller R, Bouxsein ML, Baylink DJ, Rosen CJ, Grynpas MD, Donahue LR, and Beamer WG.** Genetic regulation of cortical and trabecular bone strength and microstructure in inbred strains of mice. *J Bone Miner Res* 15: 1126–1131, 2000.
44. **Van der Meulen M.** Differential bone adaptation to mechanical loading: role of bone morphogenetic protein-5. In: *ASME Summer Bioengineering Conference, Big Sky, Montana, 1999,* p. 6889–6890.
45. **Vernikos J.** Human physiology in space. *Bioessays* 18: 1029–1037, 1996.
46. **Vico L, Novikov VE, Very JM, and Alexandre C.** Bone histomorphometric comparison of rat tibial metaphysis after 7-day tail suspension vs. 7-day spaceflight. *Aviat Space Environ Med* 62: 26–31, 1991.
47. **Wronski TJ and Morey-Holton ER.** Skeletal response to simulated weightlessness: a comparison of suspension techniques. *Aviat Space Environ Med* 58: 63–68, 1987.