

*American Physiological Society*

***Resource Book  
for the  
Design of Animal  
Exercise Protocols***

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***AMERICAN PHYSIOLOGICAL SOCIETY  
COMMITTEE TO DEVELOP AN APS RESOURCE BOOK  
FOR THE DESIGN OF ANIMAL EXERCISE PROTOCOLS***

**Kevin C. Kregel**, PhD, (*Chair*), The University of Iowa, Iowa City, Iowa; (*Chair*), Animal Care and Experimentation Committee of the American Physiological Society

**David L. Allen**, PhD, University of Colorado-Boulder, Boulder, Colorado

**Frank W. Booth**, PhD, University of Missouri, Columbia, Missouri

**Monika R. Fleshner**, PhD, University of Colorado-Boulder, Boulder, Colorado

**Erik J. Henriksen**, PhD, University of Arizona, Tucson, Arizona

**Timothy I. Musch**, PhD, Kansas State University, Manhattan, Kansas

**Donal S. O'Leary**, PhD, Wayne State University, Detroit, Michigan

**Christine M. Parks**, DVM, PhD, University of Wisconsin, Madison, Wisconsin

**David C. Poole**, PhD, DSc, Kansas State University, Manhattan, Kansas

**Alice W. Ra'anana**, American Physiological Society, Bethesda, Maryland

**Don D. Sheriff**, PhD, The University of Iowa, Iowa City, Iowa

**Michael S. Sturek**, PhD, Indiana University School of Medicine, Indianapolis, Indiana

**Linda A. Toth**, DVM, PhD, Southern Illinois University, Springfield, Illinois



# PREFACE

This resource book was developed to provide information on generally accepted practices for the design and implementation of exercise research protocols involving animals. It is intended to address experimental paradigms for the most commonly used species and models in the context of U.S. national regulatory requirements for animal welfare and to be used in the context of the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, or other relevant regulations and guidelines.

This project grew out of recommendations submitted by the Environmental and Exercise Physiology (EEP) Section of the American Physiological Society (APS) in 2001 to the APS Council. On the basis of a review of published studies involving exercise in various animal species, members of the EEP Section suggested the development of ethical guidelines to be applied in determining whether a given study should be published in APS journals. It has been a long-standing requirement of the APS “Guiding Principles for the Care and Use of Animals” that research studies published in APS journals must minimize the pain and discomfort of animals, but the Society has not offered specific guidance. APS “Ethical Policies and Procedures” are contained in the “Information for Authors” section of the Society’s journal publication instructions and available online at <http://www.the-aps.org/publications/APSEthicalPolicies.pdf>. The APS “Guiding Principles for the Care and Use of Animals” can be found online at [http://www.the-aps.org/pa/humane/pa\\_aps\\_guiding.htm](http://www.the-aps.org/pa/humane/pa_aps_guiding.htm).

The APS Council asked the APS Animal Care and Experimentation (ACE) Committee to examine the EEP recommendations in the broader context of existing regulations and guidelines. In 2002, ACE Committee Chair John N. Stallone asked Kevin Kregel as a member of both the EEP Section and the ACE Committee to organize this project. On December 3, 2002, a planning committee met at the Bethesda, MD headquarters of the APS. The group defined the parameters of the project as providing guidance rather than rules, developed a preliminary outline for the document, and identified additional areas of expertise that were needed. The NIH Office of Laboratory Animal Welfare agreed to provide support for the development of a resource concerning generally accepted standards for animal models of exercise research.

During the next several months, a committee to develop a resource for animal exercise protocols was formed, drawing upon the expertise of the planning committee and others. A first draft was prepared, with committee members contributing sections consistent with their expertise. The expanded project group held a workshop on June 18, 2003. After reviewing the initial draft, the group revised the structure of the document and made additional writing assignments.

The third content development workshop was held March 8, 2004 to review progress, discuss broad themes, and identify topics that required further attention. Kevin Kregel, Linda Toth, Erik Henriksen, and Alice Ra'anan served as editors and participated in an additional small meeting that was held on June 1, 2004 for editorial review of content. Authors were then asked to make revisions, and the document was subsequently reviewed by science writer Deborah Berlyne. A final editorial meeting was held on January 27, 2005. The document then underwent peer review, which was administered by Kenneth Baldwin. A diverse group of reviewers was chosen for both technical expertise and broad perspectives related to animal care and exercise testing issues. Comments obtained from these individuals were evaluated and integrated into the final draft of the document, which was completed during the summer of 2005.

# Acknowledgments

The authoring committee thanks the APS Council for sponsoring this project and the NIH Office of Laboratory Animal Welfare for its generous support. The committee acknowledges the contribution of Deborah Berlyne, who provided many helpful comments and suggestions, as well as the secretarial assistance of Joan Seye.

Appreciation is also extended to Kenneth Baldwin, PhD, University of California at Irvine, for oversight of the peer review process. The committee acknowledges the contributions of the following external reviewers: Paul S. Cooper, DVM, The University of Iowa; V. Reggie Edgerton, PhD, University of California at Los Angeles; John O. Holloszy, MD, Washington University; M. Harold Laughlin, PhD, University of Missouri; Roland R. Roy, PhD, University of California at Los Angeles; Kem B. Singletary, DVM, MS, The University of Iowa; and Charles M. Tipton, PhD, University of Arizona. They provided many constructive comments and suggestions. Appreciation is also extended to Drs. Edgerton and Roy for their input to the contents of this document.

A document of this magnitude requires substantial contributions from experts in many different areas. Appreciation is extended to the committee members who took part in various aspects of this project, from participation in the workshops to the drafting and editing of sections of this document. In addition, a special debt of gratitude is owed to Erik Henriksen, Tim Musch, and Linda Toth, who contributed a great deal of time and effort to all facets of this project. Finally, the committee especially acknowledges the efforts of APS Public Affairs Officer Alice W. Ra'anan, who managed the project from beginning to end and was critical to its successful completion.

Kevin C. Kregel, Chair

Committee to Develop an  
*APS Resource Book for the Design  
of Animal Exercise Protocols*





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# 1 INTRODUCTION AND OVERVIEW

## I. SCOPE OF THE DOCUMENT

The purpose of this resource book is to assist in the design and implementation of animal research protocols involving exercise. The book addresses these experimental paradigms in the context of U.S. national regulatory requirements for animal welfare.

This document was developed with several audiences in mind:

- Researchers who are new to the field
- Institutional Animal Care and Use Committees (IACUCs)
- Scientific journal reviewers and editors
- Others involved in animal research oversight

The material in this resource book is intended to provide guidance rather than constitute an exhaustive or all-inclusive set of guidelines. The authors have sought to describe “good practices” with respect to the most common kinds of exercise research that are currently being conducted and that are likely to be conducted in the foreseeable future. This resource book is intended to facilitate a problem-solving dialogue between researchers seeking to answer scientific questions regarding exercise and those responsible for animal welfare oversight.

## II. WHY STUDY EXERCISE?

Exercise is a multifactorial activity that affects virtually every organ and tissue in the body. Not only does exercise contribute many health benefits, but lack of exercise is implicated in many chronic health problems. As evidence continues to accumulate concerning the impressive range of health benefits that exercise confers (18, 34), biomedical researchers have increasingly become interested in conducting systematic studies of exercise to further define those benefits.

As improved medical treatments add years to our lives, a growing population of citizens face health problems associated with aging. For instance, obesity poses many risks, including an increased likelihood of developing diabetes, hypertension, cardiovascular disease, and muscular-skeletal disorders. The 2005 *Dietary Guidelines for Americans* urged all Americans to engage in 30 to 90 minutes of physical activity daily. Although many problems related to aging and obesity are clearly mitigated through exercise, these problems also make even modest bouts of exercise difficult to accomplish. These facts underscore the importance of learning how exercise contributes to health and of understanding as precisely as possible the duration and intensity of exercise needed to yield those benefits.

### **III. WHY USE ANIMALS TO STUDY EXERCISE?**

Most experimental paradigms involving the effects of exercise address its impacts on intact organisms, and some exercise research studies are most effectively conducted with human subjects. However, for other exercise studies, the use of human subjects is neither feasible nor desirable because these studies would involve studying humans throughout their lifetimes, which is impractical, or performing invasive procedures, which is unethical. Experimental protocols that use animal subjects are therefore developed when it would not be appropriate to use human subjects for studies of exercise's impact. Importantly, some exercise research is done with animals because it is intended to further our understanding of the biology of the animals themselves as well as to improve the health of animals.

### **IV. ANIMAL RESEARCH OVERSIGHT**

Over the past 40 years, a multifaceted system of animal research oversight has evolved in the United States in response to both scientific considerations and concerns about animal welfare. Various entities participate in this oversight process, depending on the species of animals involved, the source of research funds, where the research is conducted, and the nature of the research. Most basic research is subject to oversight from one or more of the following:

- U.S. Department of Agriculture (USDA)
- National Institutes of Health (NIH) Office of Laboratory Animal Welfare
- Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International

Each of these entities has a somewhat different approach to the challenge of simultaneously ensuring high-quality scientific research and humane treatment of research animals, but all rely on the IACUC to assume primary responsibility for ensuring animal welfare within an institution. The IACUC, in turn, must develop practices that will satisfy the requirements of the various oversight agencies. Some of these requirements are:

- **THE ANIMAL WELFARE ACT (AWA) (REGULATION BY SPECIES):** The AWA is comprised of a series of laws passed by Congress starting in 1966. For the purposes of the AWA, the term “animal” is defined as including any warm-blooded animal that is either used for, or intended for use in, research, teaching, testing, experimentation, or exhibition. The AWA definition of “animal” also contains specific exclusions (birds, rats of the genus *Rattus*, and mice of the genus *Mus*) that are bred for research. Also excluded are horses not used for research purposes and farm animals used in nonbiomedical research activities.

The USDA was designated by Congress to enforce the AWA, and the USDA’s Animal and Plant Health Inspection Service (APHIS) is responsible for AWA enforcement. The AWA is implemented through requirements and standards promulgated in the AWA regulations. Additional guidance is provided to USDA’s veterinary medical officers via various animal care policies published by APHIS. These materials are available on the USDA website at <http://www.aphis.usda.gov/ac/publications.html>.

- **PUBLIC HEALTH SERVICE (PHS) POLICY (OVERSIGHT BY FUNDING SOURCE):** The PHS Policy on Humane Care and Use of Laboratory Animals covers all vertebrate animals *including* rats, mice, and birds when the research is funded by PHS agencies. All NIH-funded research must comply with the PHS Policy, which includes adherence to the National Research Council’s *Guide for the Care and Use of Laboratory Animals*. Some other government agencies also require their grantees to comply with the standards set forth in the PHS Policy. The PHS Policy is available online at <http://grants2nih.gov/grants/olaw/references/phspol.htm>.
- **AAALAC (ACCREDITATION BY PROGRAM):** AAALAC International is a private, nonprofit organization that provides voluntary accreditation of animal care programs. Many major research

institutions participate in the AAALAC accreditation program. AAALAC accreditation requires adherence to the requirements set forth in the National Research Council's *Guide for the Care and Use of Laboratory Animals* as well as compliance with the AWA and other relevant laws as necessary. Information about AAALAC is available at <http://www.aaalac.org>.

- **THE GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS (SETTING PERFORMANCE STANDARDS):** The *Guide for the Care and Use of Laboratory Animals* is a publication of the National Research Council's Institute for Laboratory Animal Research (1). Its seventh edition was published in 1996 and incorporates changes reflecting the most recent changes to the AWA and the PHS Policy on Humane Care and Use of Laboratory Animals. The *Guide* is intended to "assist investigators in fulfilling their obligation to plan and conduct animal experiments in accordance with the highest scientific, humane, and ethical principles" (*Guide*, Preface, p. ix). The *Guide* contains recommendations that are "based on published data, scientific principles, expert opinion, and experience with methods and practices that have proved to be consistent with high-quality, humane animal care and use" (*Guide*, Preface, p. ix). One of the hallmarks of the *Guide* is its acceptance of "performance standards" as its evaluation criteria: "The *Guide* charges users of research animals with the responsibility of achieving specified outcomes but leaves it up to them how to accomplish these goals" (*Guide*, Introduction, page 3). The use of performance goals is important in designing scientific studies because it allows for flexibility when "acceptable alternative methods are available or unusual circumstances arise" (*Guide*, p. 3).

One of the key roles of the IACUC is to review and approve protocols before the research begins. Animal welfare questions considered in this review may include:

- Is the rationale for the proposed use of animals in this research adequate to justify the work?
- Are the species and number of animals appropriate for producing meaningful data?
- What provisions consistent with sound research design will the investigator make to limit discomfort, pain, and injury to the animals?

- What analgesic, anesthetic, and tranquilizing drugs consistent with sound research design can be used to minimize pain or discomfort to the animals?

We hope that this resource book will promote efforts to arrive at satisfactory responses to these questions.





# **GENERAL ASPECTS OF ANIMAL CARE AND THE DEVELOPMENT OF ANIMAL USE PROTOCOLS**

## **I. OVERVIEW**

In this chapter, we discuss several issues related to the development of research protocols for exercise studies involving animals. First, we discuss the criteria to use in selecting appropriate animal models for exercise research. We then describe several issues that must be discussed in the research protocol. Finally, we consider issues that require particular attention in exercise studies, such as how to minimize the stress response and how to manage animals with specific health concerns.

## **II. SELECTING ANIMAL MODELS FOR EXERCISE RESEARCH**

Investigators must consider a wide variety of factors when selecting the best animal model for their research. An obvious choice is the animal species and model used historically for the same type of research, but currently there are an increasing number of animal models available to investigators. Many of these are genetically modified, resulting in alternative models that may offer better choices. Scientific and practical considerations that influence the choice of animal model are listed in Table 2.1. In addition, regulatory agencies, funding agencies, and IACUCs may require the use of the least stressful model, including, where appropriate, in vitro and human models, that is compatible with experimental goals. Studies addressing human health issues require an understanding of the normal human responses to acute and chronic exercise and information about how these factors are reflected in the animal model (2).

**Table 2.1**

<b>Considerations in Selecting an Animal Model for Exercise Research</b>	
<b>Scientific</b>	<b>Practical</b>
Appropriateness of the system for testing the proposed hypothesis	Availability of the animal, including appropriate age, sex, reproductive status, genetic characteristics, and numbers
Responses of the animal to necessary surgical and experimental procedures	Availability of facilities and husbandry care necessary for appropriate housing
Number of animals needed, based on sound statistical design	Availability of appropriate equipment and expertise for exercise training, surgery, and other necessary experimental activities
Experimental requirements, such as biological age or genetic background	Any special animal care requirements and necessary housing or testing equipment

### **III. STUDY DESIGN CONSIDERATIONS**

#### **A. Protocol Development**

Animal experiments designed to test the impact of exercise on physiology and health outcomes entail several complex issues that should be addressed when choosing the optimal exercise protocol. In this section, we review issues that arise in the development, review, and execution of exercise research using animals. The recently published *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (2) contains information and guidance on these issues that are relevant to many different types of research, including exercise research. Readers should explore that text for pertinent information.

As with all animal-based research, a team approach to developing performance-based design and implementation standards is key to success. The research team, veterinary staff, and IACUC should to work together to develop the animal care and use protocol. Good communication can allow the IACUC to understand the scientific issues, common methodologies, and

interpretational constraints that impact exercise research. Concurrently, the principal investigator (PI) should recognize the importance of compliance with the regulations and policies that govern animal care and use and the role of the IACUC in achieving institutional compliance with regulatory mandates. Open dialogue and professionalism during the protocol development and review process can promote a functional balance between good science and good animal care, achieving the goals of all parties.

The challenge in exercise protocols is twofold. First, it is necessary to identify reliable experimental and performance criteria that will not unduly impact the study's scientific goals. Second, investigators must ensure that humane procedures are utilized when acute or chronic exercise protocols are conducted on animals. Potential conflicts concerning when to terminate an exercise session, remove an animal from the study, or use euthanasia can be avoided by developing quantifiable or otherwise objective criteria for each of these actions. Different criteria may be applied during the training or conditioning period and during acute or chronic exercise periods. As part of the protocol development process, the PI, the IACUC, and laboratory animal veterinarians should develop an intervention plan to prevent animal distress before a crisis occurs. The intervention plan should also specify an unambiguous line of authority within the laboratory for addressing animal distress.

## **B. General Considerations for Exercise Study Design**

***IACUC review.*** The PI and the IACUC should maintain flexibility and a rational, open attitude regarding interpretations, recommendations, and compliance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* (the *Guide*) and other policies and regulations. A conscientious and thoughtful process of protocol development and outcome assessment, with documentation, is crucial. Examples of issues that require consideration, discussion, justification, and perhaps compromise are discussed briefly below.

***Animal numbers.*** Regulatory agencies and sound scientific practice require that protocols use the minimum numbers of animals consistent with sound statistical design. The Institute for Laboratory Animal Research (ILAR) document *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (2) contains a useful appendix that addresses important issues related to sample size determination for animal research.

Both scientists and IACUC members are encouraged to consult this document or similar materials to determine the appropriate number of animals needed for an experimental protocol. Statistical power calculations should also be considered when evaluating sample sizes. In some cases, the appropriate number of animals should include replacement animals for subjects that are either unwilling or unable to perform an exercise protocol.

***Animal use.*** All facets of animal use must be specified in the animal protocol. Some are common to all animal protocols, such as requirements for a scientific rationale for the species used, numbers of animals needed, method of euthanasia, and potential need for decapitation or cervical dislocation in unanesthetized animals.

Other aspects of the protocol are specific to studies incorporating exercise regimens. These should be considered in planning such studies and must be described in the animal protocol. These aspects include the need for forced (as opposed to voluntary) exercise, exercise to exhaustion, and imposed workloads; the duration and intensity of individual bouts of exercise; the number of exercise bouts the animal will experience; the time interval between repeated bouts; the duration of the study; the use, intensity, and frequency of aversive stimuli to maintain performance; and the need for special caging or restraint. A proposal to house or exercise animals under environmental conditions that deviate from ranges provided in the *ILAR Guide for the Care and Use of Laboratory Animals* requires specific justification. Procedures for animal familiarization with the exercise equipment and environment should be fully described. The protocol should also specify animal monitoring procedures that will be employed both during exercise bouts and during recovery. Personnel responsible for monitoring animals and the criteria that will trigger premature termination of an exercise session must also be specified.

All exercise protocols have the potential to cause inadvertent injury to experimental animals. If automated exercise equipment is used, animals should initially be trained at low speeds, inclines, and durations. These parameters can then be increased gradually as animals gain stamina and experience. Similar considerations apply to protocols that use swim training. Safety equipment that may reduce or prevent injury during exercise should be described. Potential adverse consequences associated with exercise protocols—such as drowning, physical injury, or increasing tolerance to aversive stimuli—should also be clearly identified in the protocol, and

procedures for dealing with these circumstances should be specified. Continuous monitoring should always be provided when historical data are not available and is preferable when automated equipment is used.

Food and water are used as motivators in some types of exercise regimens. When growing animals are required to exercise for a food reward, the animals may perform the minimum amount of exercise needed to satisfy minimal caloric requirements and so may lose weight or grow less rapidly than nonexercised animals; such effects can be important if data are expressed in relation to body weight (e.g., muscle weight/body weight) (238). For additional information in restriction/reward protocols, see *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (2).

A relatively new and expanding activity in the field of exercise science is strength training, which is the desired outcome of much rehabilitation therapy and is usually achieved through progressive resistance (weight) training and the concomitant development of muscle hypertrophy (238). Several approaches have been used to induce muscle hypertrophy in a variety of animal species: resistance training in conscious animals (i.e., weight lifting), electrical stimulation of muscles in anesthetized animals, compensatory overload induced by tenotomy or surgical ablation of synergistic muscles, imposition of chronic stretch via weight application or casting, and wheel running with high resistance (172, 238, 369). Each general category has particular advantages and disadvantages with regard to interpretational confounds and similarities to strength training in humans (238). Refinements should be developed to minimize the imposed workload while achieving satisfactory conditioning.

***Surgery and other procedures performed under anesthesia.*** As with all protocols, surgery must be justified and performed under anesthesia and must include regimens for postsurgical analgesia and monitoring. Omitting analgesia for procedures that would normally be deemed painful for humans requires scientific justification that includes literature citations, if available. Personnel who will provide nursing care should be identified, and a schedule of observation and/or treatment should be developed. The PI should specify the interval between surgical interventions and initiation of an exercise protocol and justify this interval by showing that it will provide time for adequate recovery from surgery.

The standard and regulatory benchmark for whether or not analgesics are necessary is the analogous human condition: If a human patient would request or receive analgesia after a similar procedure, the animal should receive a comparable analgesic therapy. When performing survival surgery on animals, investigators must provide appropriate postoperative analgesia as determined by the facility veterinarian unless omission is justified for scientific reasons and approved by the IACUC. Investigators should fully describe analgesic regimens in the methods section of publications resulting from the research. If analgesics cannot be used for scientific reasons, this rationale or justification should be clearly explained in the methods section, with appropriate references.

***Personnel, animal monitoring, and records.*** Trained personnel must be available to observe animals that are performing an exercise protocol. This requirement is particularly important during the early phases of a conditioning program, near the end of individual training sessions, and during sessions in which performance requirements are increased. All research staff should be familiar with the normal appearance and behavior of the species of animal used in the experiments so that they can recognize problems immediately. If questions arise regarding animal health, the veterinary staff should be consulted regarding treatment of the animal or removal of the animal from the study. Early intervention and treatment by the veterinarian may prevent the need to remove the animal from the study permanently.

Good record keeping is a crucial component of ensuring good animal care, complying with regulatory requirements, and conducting good science. Animals on exercise regimens require regular (perhaps daily) record entries to document their performance level and other aspects of their condition. The level of detail necessary for such entries will vary with the nature of the protocol. Voluntary wheel running, for example, may require little or no comment after animals become experienced with the equipment. However, a reduction in the amount of running may signal a problem with the equipment, the animal, or the environment and therefore warrants a detailed entry. More complex protocols, such as those requiring an imposed workload or work to exhaustion, require more comprehensive notations regarding the animal's performance and recovery.

Automated animal exercise equipment sometimes applies mild aversive stimuli, often in the form of mild electric shocks, to maintain performance. Although the number of shocks experienced by trained and conditioned

animals is typically low, monitoring the frequency or number of shocks animals experience and the pattern of shock administration during the training session is an essential part of animal monitoring. Changes in the frequency of administered shocks can signal animal exhaustion, injury, or equipment malfunctions. Careful selection of animal subjects and gradual training should be employed to minimize the number of aversive stimuli experienced by each subject. However, the maximal permissible intensity and frequency of shock delivery, as well as the need for motivating aversive stimuli at all, depend on the study design and the specific experimental goals. Some experimental or design requirements (e.g., exercise in obese or sedentary animals, exercise at a high intensity, exercise to a predetermined targeted physiological change, exercise to exhaustion) may require relatively frequent stimulation. IACUCs, PIs, and veterinarians should collaboratively review specific protocols to determine an acceptable limit for application of aversive stimuli.

Studies that require exercise to exhaustion require special consideration. The need for such extreme effort by the animal must be carefully defined and justified, and end points must be clearly established and well defined. Specific behaviors, circumstances, or physiological markers must be established to alert the observer that the trial must be terminated. Continuous animal observation is essential near the time of the expected development of animal exhaustion. In all cases, accurate records of test conditions and of performance should be maintained for each animal. Such records will allow day-to-day adjustment of test parameters, if warranted by the animal's condition or ability.

***Health problems.*** PIs must provide criteria for temporary or permanent removal of animals from a study because of health problems. Numerous signs can indicate that an animal is developing health problems, which may or may not be related to experimental procedures, that may affect performance. Changes detected in an animal's demeanor or willingness to perform may be the first signs of a health problem. Common signs of pain, illness, or distress include decreased appetite, weight loss, decreased spontaneous activity, guarding of specific areas of the body, abnormal gait or posture, porphyrin rings around the eyes, changes in bowel or bladder habits, and irritability. Signs of more severe illness include decrease in body temperature, weak pulse, or decreased respiration. If such changes occur, the researcher should promptly notify the veterinarian so that the animal can be evaluated fully. These signs can be used as criteria for temporary or permanent removal of an

animal from study. PIs, IACUCs, and veterinarians are encouraged to be creative, flexible, and compassionate in developing these criteria.

Sanitizing devices used for exercise or learning paradigms, although important in all cases, may be particularly crucial for animals that are physically or physiologically impaired because such impairments may contribute to subtle but real defects in host defense against opportunistic organisms in the environment. In addition, devices that are used for exercising multiple animals without intervening sanitation present a likely locus for the transmission of infectious diseases that may be present in the colony.

***Stopping an exercise session.*** The PI and the IACUC should determine a humane end point for removing animals from a test situation. This end point must be specified and approved in the animal use protocol. Reasons for stopping an exercise session prematurely include accidental injury, fatigue (potential indicators include increased heart rate, high lactic acid levels, and inability to perform), elevated colonic temperature, unexpected adverse effects, behavioral issues, and poor performance caused by the animal's unwillingness to exercise.

***Removal of an animal from an exercise study.*** Animals that are trained and conditioned for exercise studies are valuable resources and often must be maintained over long periods of time. Numerous events—including infectious disease and trauma—can, at least temporarily, necessitate removal of an animal from an exercise study. An animal that has recovered from injury or disease and is released by the veterinarian can reenter the study if feasible. The protocol should specify the circumstances under which animals should be temporarily removed from exercise studies and the criteria for returning them to the study.

An animal should be removed from the study if it becomes permanently unable to perform satisfactorily. Inciting causes can be either physical impairment or poor temperament. Animals that require excessive motivation (e.g., aversive stimuli) to exercise should also be removed from a study. The protocol should specify the general circumstances under which permanent removal will be necessary.

***Reuse of animals.*** In some circumstances, euthanasia of instrumented and trained animals may not be necessary at the end of a study. Depending on the condition and prior use of these animals, the IACUC may allow them



to be used in a subsequent study. Reuse that requires additional (i.e., multiple) major survival surgeries requires specific justification and IACUC approval.

## IV. ANIMAL STRESS AND HUMANE STUDY DESIGN

### A. Stress and Exercise

Acute activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis is a common, adaptive, and potentially necessary feature of exercise training (46, 118, 341). These physiological changes are often described as a stress response. There is a large body of literature indicating that acute and regulated activation of the stress response facilitates many aspects of the fight-or-flight response (336, 343). Stimulation of the sympathetic nervous system and the HPA axis, for example, increases blood pressure and blood flow to active muscle, decreases blood flow to digestive organs, mobilizes energy production and utilization, and potentiates some aspects of the immune response (76, 104, 105, 129).

The difficulty for physiologists with respect to exercise protocols is that chronic or prolonged activation of the stress response can adversely affect animal well-being and confound interpretation of the outcome variables of interest. For instance, chronic stimulation of sympathetic nervous system and HPA responses can directly increase vascular stiffness (174), suppress the immune system (75), decrease viral resistance (346), induce ulcers (299, 343), and damage areas of the brain (337).

A fundamental issue for exercise studies involving animals is selection of an exercise protocol that will produce the desired physiological changes or adaptations without producing the confounding negative consequences of a nonspecific or chronic stress response. Making this determination can be difficult. In most cases, the investigator must choose an exercise protocol that balances the positive and negative physiological consequences. This decision can be facilitated by answering the following questions:

1. What is the minimal amount of exercise (intensity, duration, frequency) necessary to produce the anticipated or required changes in outcome measures of interest? The “dose” of exercise needed to produce positive adaptations in physiology and health is an empirical question that is often specific to the system of interest (e.g., changes in body composition, skeletal muscle adaptations, cardiac muscle adaptations, changes in metabolism).

2. What type of exercise will best elicit the requisite changes in physiology while minimizing negative and confounding consequences of chronic activation of the stress response?
3. Has the protocol been designed to maximize perceived behavioral control and minimize the novelty of the exercise apparatus and procedures?

## **B. Subject and Stressor Characteristics**

Many factors, including characteristics of both subject and stressor, affect the development of a stress response in association with an event or activity and the consequences of that response to the organism. General conclusions regarding the impact of such factors may not reflect all situations. Characteristics that can influence the response to exercise include age, gender, strain, health status, and housing conditions. Characteristics of an exercise protocol that can influence the stress response include intensity, frequency, and duration. The protocol director should carefully assess subject and stressor characteristics when choosing an animal model of exercise.

Exercise protocols should be designed to test the desired physiological adaptations without producing confounding nonspecific chronic activation of the stress response. Other features of exercise protocols that are less commonly considered but are equally important include perceived behavioral control and novelty. The literature contains many examples in which loss of behavioral control over the environment is highly stressful (243). In addition, animals exposed to a novel environment often show activation of the stress response (130, 348).

Researchers can reduce activation of the stress response by maximizing the animal's perceived behavioral control and minimizing the novelty of the exercise situation. For instance, perceived behavioral control can be maximized by, for example, training the animal during the natural active phase of its circadian cycle (304). Perceived behavioral control can also be maximized by allowing the animal to choose the timing, speed, and duration of the exercise bout, as in voluntary wheel running. However, under some experimental circumstances (e.g., during food restriction), even wheel running can cause chronic stress, as evidenced by the development of ulcers, splenic and thymic hypotrophy, and adrenal hypertrophy (137, 275).

To minimize the novelty of the exercise, investigators should expose the animals to the exercise apparatus repeatedly before beginning actual

training. For example, for treadmill training, repeated exposure to handling and the treadmill apparatus at the same time of day and by the same personnel who will conduct the actual training sessions will greatly reduce the stress response triggered by a novel environment and activity.

### C. Indexes of Acute and Chronic Stress Responses

Acute activation of the stress response is a normal and adaptive attempt of the body to maintain or restore homeostasis. In fact, many so-called stress responses are normal facets of the physiological response to acute exercise.

The physiology of the stress response is complex and can be assessed at molecular, cellular, physiological, and behavioral levels. Indexes of stress that are most useful in the design of animal protocols are those that can easily be assessed in the performing animal. The indexes listed in Table 2.2 are just a small sample of many possibilities but are highlighted here because these changes may develop in association with exercise and can be monitored and measured rather easily in blood or peripheral tissues. Some signs of chronic stress, such as adrenal enlargement or ulcers, are not easily assessed in live animals. In such cases, carcasses can be evaluated after euthanasia and the exercise protocol can be adjusted for future subjects if necessary.

**Table 2.2**

<b>Peripheral responses to acute stress</b>	<b>References</b>	<b>Peripheral responses to chronic stress</b>	<b>References</b>
Catecholamines increased in blood	249	Adrenal enlargement	137, 269, 275, 343
Elevated concentrations of circulating cortisol or corticosterone	46, 118	Thymic involution	137, 269, 275, 343
Altered circulating cytokines (e.g., interleukin-6)	53, 251, 357, 398	Splenic hypertrophy	137, 269, 275, 343
Levels of extracellular heat shock proteins	45, 95	Ulcers	343
Neutrophilia	47, 104	Decreased corticosterone binding globulin	269, 355
		Suppressed T and B lymphocyte function	138, 154, 269

## **V. WORKING WITH COMPROMISED ANIMALS: ANIMAL MODELS OF DISEASE THAT LIMIT EXERCISE PERFORMANCE**

### **A. Special Considerations for Training Compromised Animals**

Many experiments are designed to assess the beneficial or detrimental impact of exercise in animals with certain primary disease conditions, such as hypertension, heart failure, obesity, diabetes, and neuromuscular disease. This section reviews considerations that apply to animals in which physiological impairments are induced as part of the research protocol. The disease conditions presented are not intended to be inclusive of all research models that could be incorporated into this category, but serve instead as representative examples of special considerations that may apply if an animal's capacity to exercise is compromised or limited.

All studies that require forced exercise in animal models of human disease should be carefully justified with regard to the advancement of biomedical knowledge, potential benefits of the work, avoidance of duplication, and scientific merit. In addition, studies that employ physical conditioning require appropriate attention to adaptation of the animal to the training situation, to gradual conditioning of the animal to develop stamina, and to close observation of the animal during the exercise period. These considerations are particularly crucial for animals with any type of overt physical or physiological impairment. Impaired animals may require more gradual familiarization with automated training devices than healthy animals. For example, a conditioning schedule for treadmill exercise of an impaired animal might include lower initial speeds, inclines, and durations, with a more gradual rate of increase in these parameters that is closely linked to the animal's performance.

Knowledgeable personnel should closely monitor impaired animals during training and exercise sessions, particularly in the early phases of a conditioning program, near the end of training sessions, and during sessions in which performance requirements are increased. In some cases, remote monitoring systems such as closed-circuit cameras may be indicated. Monitoring the frequency, number, or pattern of shocks that animals experience during training sessions can provide an index of their ability and state of conditioning, particularly compared to normal animals. Behavioral or physiological markers may be identified that can alert the observer that

the trial must be terminated or the demands reduced. Accurate records of test conditions and performance should be maintained for each animal to permit day-to-day adjustment of test parameters, if warranted by the animal's condition or ability.

## **B. Disease Models that Limit Exercise Capabilities**

A wide variety of disease models are likely to limit exercise capabilities or exacerbate an animal's clinical condition. Common examples include models of cardiovascular disease (e.g., pharmacological or surgical impairment of cardiovascular functional or homeostatic capacities, heart failure, hypertension, ischemia-reperfusion, peripheral arterial insufficiencies, atherosclerotic defects), respiratory impairments (e.g., emphysema), hematologic (e.g., anemia) and neuromuscular diseases, endocrinopathies (e.g., obesity, diabetes, hypo- or hyperthyroidism), and orthopaedic problems (including those induced by surgery or nutritional status).

Genetic modifications that promote the "spontaneous" development of disease (e.g., diabetes or cardiomyopathy in rodents) may require establishing temporal and functional benchmarks related to the expected development and progression of the disease condition. For example, dystrophic animals (*dy/dy* mouse, *mdx* mouse, and dystrophic hamster) generally run voluntarily for only about half the distance as control animals, and muscle resistance to fatigue in these animals decreases substantially with age (391). Physiological variation and impairments may also be related to such factors as aging and thermoregulatory demands and capabilities (209, 210). The design of exercise paradigms used in the context of such disease states requires forethought and flexibility to allow adjustments that might be necessary as the condition's duration and severity change.

Using water or food as motivators and imposing restriction paradigms in association with exercise can exacerbate physiological impairments in animals with various medical conditions. For example, diabetic animals may become dehydrated if water availability is restricted during exercise. Restriction paradigms that interfere with homeostatic adjustments to a physiological defect or exacerbate a condition should be avoided unless no alternative is available.

## VI. IMPACT OF SURGERY ON EXERCISE

### A. Surgical Procedures on Animals Used in Exercise Studies

Surgical placement of instrumentation or excision of tissue (e.g., biopsies) is frequently necessary to collect physiological and biochemical data from animals used in exercise studies. Careful attention to selection of anesthesia and instrumentation and allowing adequate recovery from anesthesia and recuperation after surgery before experimental use are critical to the collection of valid data. In one study, for example, aortic catheterization was associated with a 56% reduction in liver glycogen 24 hours after the procedure as compared to untreated animals, with a variable recovery period of 2 to 8 days (267). Because of the large interanimal variation in recovery times, assessment of recovery of individual animals based on attainment of a normal ratio of food intake to body weight predicted normalization of liver glycogen content more accurately than reliance on a fixed 6-day recovery time (267). Instrumented rats regain a cardiovascular steady state within 1 hour after halothane anesthesia and are thereafter stable for at least 5 hours (103). In contrast, heart rate and other cardiovascular and metabolic parameters require 6 to 12 hours to stabilize after methoxyflurane anesthesia of mice (73). Acute instrumentation produces a significant reduction in maximal aerobic power ( $VO_{2max}$ ) and a reduction in body weight (110, 121, 122, 152). Therefore, it is recommended that the recovery period not be terminated until an animal has returned to within 10% of its presurgical body weight.

### B. Selecting Anesthesia for Animals in Surgery

Anesthetic or other drugs used before collection of tissue for biochemical analysis can influence several parameters, including arterial blood gases, acid-base status, plasma hormone levels, and glycogen content in liver and skeletal muscles (282). Investigators should carefully consider the impact of anesthesia on the animal and the dependent variables. A thorough literature review can provide critical information. In rats, for example, neither halothane nor ketamine-xylazine differentially influences liver and skeletal muscle glycogen content, making them appropriate for studies that require use of anesthesia before collection of tissues for glycogen measurement (282). However, pentobarbital anesthesia differentially reduces liver glycogen phosphorylase activity in rats depending on the route of administration (259). In rats, anesthetics such as pentobarbital obscure the

effects of exercise on acid-base status, but decapitation negates exercise-induced changes in muscle metabolites (109). Compared to isoflurane and sevoflurane, pentobarbital administered before heart excision increases lactate levels and alters functional characteristics and stabilization during reperfusion in an *ex vivo* working heart model (291). In contrast, enflurane increases the ratio of lactate to pyruvate in heart and liver of rats subjected to hemorrhage as compared to pentobarbital or isoflurane (188). In mice, cardiovascular and metabolic effects of anesthesia normalize within 6 hours after methoxyflurane but are altered for over 24 hours after anesthesia induced by 2,2,2-tribromoethanol (73).





# 3 CHAPTER 3. EXERCISE PROTOCOLS USING RATS AND MICE

## I. OVERVIEW

This chapter focuses on issues related to exercise modalities using rats and mice, the species most frequently used in exercise studies. These exercise modalities include treadmill running, voluntary wheel running, and swimming. Discussed in sections devoted to each of these two species are: 1) the rationale for using each exercise modality, 2) the issues to consider when using that exercise modality (e.g., guidelines for the speed, intensity, and duration of exercise), 3) an overview of the physiological outcomes that confirm that the respective exercise modality has produced the anticipated acute and/or chronic adaptive responses, and 4) a brief description of concerns related to the use of that exercise modality. This latter area includes information about potential limitations of that particular type of exercise, experimental conditions (such as environmental conditions under which these animals are exercised) that can affect the exercising animal, and the use of these exercise modalities in physiologically compromised rats and mice (such as aged animals and animals displaying specific pathophysiological conditions, such as hypertension or obesity).

## II. EXERCISE MODALITIES IN RATS

### A. Treadmill Running in Rats

#### 1. Rationale for the use of this exercise modality

Treadmill running has been used extensively over the past four decades to study behavioral, physiological, biochemical, and, more recently, molecular responses to both acute exercise stress and chronic exercise training. Although investigators have used a wide variety of species—including dogs (277), rabbits (77), cattle (107), and ducks (192)—for treadmill running studies, they have used rodents in most of these studies. Therefore, this section focuses on rats and treadmill running.

Treadmill running has the distinct advantage over other forms of exercise, including spontaneous wheel running and swimming, that the total amount of external work done by the rat can be easily calculated (41). In addition, if the metabolic rate (oxygen uptake and carbon dioxide production) of the animal is determined during both submaximal and maximal exercise workloads, efficiency can be calculated and the responses to acute exercise can be defined relative to the rat's maximal aerobic power ( $\text{Vo}_{2\text{max}}$ ) (25, 40, 41). Another advantage of treadmill running is that the investigator can control both exercise intensity and duration. This enables the investigator to closely examine the factors contributing to exercise performance while the rats perform under well-defined experimental conditions. The treadmill exercise modality also allows for the determination of kinematics (e.g., easy to videotape, more consistent locomotion) more readily than other exercise modalities.

This animal model also allows investigators to examine the determinants of exercise performance with invasive techniques in both acute and chronic exercise settings. Finally, by using different rat models of pathology (e.g., obesity, diabetes, chronic heart failure, hypothyroidism), investigators may be able to unravel the physiological, biochemical, and molecular mechanisms that contribute to the large decrements in exercise capacity commonly associated with these different disease states.

Although there are many advantages of using treadmill running in rats, this exercise modality does possess a number of disadvantages compared with other forms of exercise. For example, treadmill running may be construed as a form of forced exercise in which the animal does not have a choice of participating in the activity. Because of this, noxious stimuli (e.g., electric shock and bursts of high-pressure air) may be needed to motivate the animals to exercise. As a result, this exercise modality does not represent the normal physical activity patterns of the domesticated or nondomesticated rat. Rats may also display a normal type of "stop and go" running activity on a treadmill. This type of running behavior primarily occurs in naive rats when they are first exposed to treadmill running. However, this type of "stop and go" running behavior may disappear with repeated bouts of exercise, as the rat becomes familiar with the activity.

Another concern is that rats participating in treadmill running studies may risk developing certain injuries (e.g., breaking toenails or injuring their paws). Many of these injuries can be avoided by trimming the rat's toenails

and/or applying a small amount of cyanoacrylate (i.e., superglue) to its cuticle. Constant surveillance of the animals is necessary to prevent injury. Because treadmill running is a form of forced exercise, it may require aversive stimuli to keep the animal running. An additional consideration is that commercial treadmills can be expensive and may not provide the flexibility in treadmill design (e.g., speeds and grades) needed for certain experimental protocols.

## 2. Appropriate use of this modality

**Treadmill design.** The design of the equipment is important because it influences the animal's running behavior and, as a result, may determine the success or failure of an exercise study. Accordingly, recommendations are provided based on data from the literature regarding treadmill specifications to increase an experiment's likelihood of success.

Rats running on the treadmill must be able to maintain good traction while walking or running, to prevent slipping (330). The surface of the treadmill belt should not be porous and should be soft enough to minimize toenail and foot problems that may arise from daily bouts of exercise training. Most treadmill belts consist of smooth rubberized surfaces that are easy to clean and disinfect. These types of belts usually wear well, with minimal breakage, and rarely need to be replaced during the lifetime of the treadmill.

Because the treadmill is motor driven, the investigator should make sure that the motor can produce a wide range of revolutions per minute (rpm) to provide a significant range of treadmill speeds. Rats can and will run in excess of 70 m/min (11, 281), and therefore the investigator also needs to make sure that the treadmill's maximum speed is sufficient for the planned studies. One common problem with some commercially built treadmills is that even though their motors have the rpm range needed for the study the motors may not possess enough horsepower to maintain the rpm needed when a significant load is added to the treadmill belt. This problem can arise when investigators want to measure a rat's metabolic rate and add a Plexiglas chamber to sit and slide on the treadmill belt as the animal exercises (41). Therefore, the motor must have enough horsepower to handle the extra weight without affecting treadmill performance.

The natural running behavior of naive rats (who have not been exposed to treadmill running) includes periods of stopping and sniffing (392). Because of this type of "stop and go" running behavior, the length of the treadmill

belt and the amount of room the rat is given to run are important. Wisloff and colleagues (392) developed and used a treadmill with running lanes that were 70 cm in length, allowing rats to avoid unnecessary contact with an electrical grid placed at the rear of each lane. By comparison, Bedford and colleagues (25) used a treadmill belt length of 39 cm, but the treadmill was housed in a metabolic chamber such that oxygen uptake ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) could be measured while the animals ran at varying speeds and grades. Their results suggest that a treadmill belt length of 39 cm is long enough for most experimental purposes. Longer treadmill belt lengths accommodate some degree of “stop and go” running behavior, as well as minimizing the animal’s contact with aversive stimuli placed at the rear of the treadmill lane.

Treadmills used with rats should be equipped with some type of mechanical device that enables the investigator to set the treadmill belt to an upward and/or downward incline. In most studies, rats are required to run up grades of 0 to 20%. However, rats can run up inclines of 30° or grades of 35% (25, 392) and down inclines of 16° (12). The ability to change treadmill grade along with speed enables the investigator to increase or decrease exercise workloads to varying degrees. Moreover, these two factors affect the recruitment pattern of the hindlimb musculature during locomotion, which could be important to the investigation at hand.

Many treadmills are equipped with electric grids at the rear of the treadmill lanes that provide an aversive stimulus to keep the animals exercising. The electric grid should be noxious enough (i.e., high enough voltage) to provide a significant incentive to keep the rat exercising, but at the same time it should not harm the animal. If excessive electric shock (e.g., more than four times a minute) must be delivered to the animal to elicit compliance with the exercise intensity, the investigator should seriously consider decreasing the exercise workload. If this approach is unsuccessful in maintaining compliance at any exercise intensity, the animal should be removed from the study. Both metal prod and grid designs are effective in keeping the animal running. Metal prods or grids have a variable power source, and 10 to 30 volts of electricity is sufficient to motivate the animals to run. Electric grids used with rats running on a treadmill should consist of an electric source of very low amperage (0.5 amps). Moreover, the amount of voltage delivered to the grid should be kept to the minimum needed to keep the animal running. Bedford and colleagues (25) used an electric grid that contained 12 separate 2.5-mm-diameter steel electrodes spaced 1 cm

apart. If the rat ventured to the rear of the treadmill and actually stepped off the belt and onto the grid, it was subjected to an electric shock. In contrast, Brooks and White (41) used an electric grid consisting of metal prods that protruded 4.5 cm in the rear of the treadmill lane. Sonne and Galbo (353) used a displacement plate at the rear of the treadmill lane that turned on a microswitch that produced a varying electric shock of 0 to 40 volts to the animal's tail. Thus a wide variety of electrical grids have been developed to keep the animals running.

In many situations, nonpainful stimuli are sufficient to motivate rats to continue running on the treadmill. Specifically, the sweeping use of a bottle brush on a tail that extends near the rear of the treadmill (370, 371) or the application of high-pressure air to the animal's hindlimbs (112, 147, 263) has been used in performance studies, in which rats run to the point of fatigue, or in training studies, in which rats are exposed to repeated bouts of daily exercise. One disadvantage of using high-pressure air is that this stimulus cannot be used when determining the  $\dot{V}O_2$  and  $\dot{V}CO_2$  of the animal because precise measurements of airflow through the metabolic chamber are required. Adding high-pressure air into the chamber makes an accurate measurement of metabolic rate virtually impossible. Under these circumstances, electric shock should be used to motivate the rat to keep running.

***Familiarization with treadmill running.*** Although rats are excellent runners in open territory, getting them to walk or run on a motorized treadmill can be challenging. In fact, as many as 10% of the rats purchased from commercial vendors refuse to walk or run on a treadmill, and these animals must be removed from exercise studies (25, 84, 176, 202). To minimize the number of rats classified as "nonrunners," investigators should introduce the rats to treadmill exercise gradually. Familiarization will help the animals become proficient runners and minimize the potential for foot injuries that can occur with chronic exercise training. Foot injuries should be evaluated and treated under the supervision of a veterinarian. If a rat's foot injuries persist for more than a few days, the animal should be removed from the daily training regimen to permit recovery. After being deemed healthy by the veterinarian, the rat may be returned to the exercise training requirement.

Familiarizing rats with treadmill running can take as little as 5 days or as long as 2 weeks (23, 140, 392). During the familiarization period, the rat becomes proficient at exercising within a confined treadmill lane. Most investigators use repeated bouts of short-duration exercise (usually 5 min

per session or less) and varying speeds to ensure that the rats become excellent runners (51, 148). The frequency and duration of these running sessions must be kept to a minimum to avoid a significant heat shock protein (stress) response or an aerobic training effect (19, 84, 263).

The use of a positive reinforcement during the period of familiarization can be effective in producing extremely proficient runners. Wisloff and colleagues (392) rewarded each rat with 0.5 grams of chocolate at the conclusion of each exercise session and found that rats typically jumped out of their transfer cages and onto the treadmill after 2 weeks of familiarization. When these investigators used this type of positive reinforcement, they found that none of the rats had to be excluded because of noncompliance. Moreover, they found that the amount of shock stimuli needed to keep the rats running was minimized in these well-conditioned animals (392). However, the use of food (especially high-calorie or high-fat foods) as a positive reward may not be warranted in some investigations that pertain to diet issues.

### 3. Factors influencing performance

**Measuring exercise performance.** Typically, investigators assess exercise performance or fitness in rats running on a treadmill by assessing  $\text{VO}_{2\text{max}}$  or endurance capacity. Assessing  $\text{VO}_{2\text{max}}$  requires the use of a plastic chamber or mask to determine the animal's metabolic rate (25, 41, 121, 276). To evaluate endurance capacity, an animal is run to the point of fatigue under carefully controlled experimental conditions. This test is usually performed at designated submaximal workloads.

**Measurement of  $\text{VO}_{2\text{max}}$ .**  $\text{VO}_{2\text{max}}$  for rats running on a motorized treadmill is normally defined as the point at which  $\text{VO}_2$  does not increase, even though further increases in external workload are imposed on the animal (345). Some investigators use progressive exercise tests to meet these criteria (41, 121, 276, 392). However, others report that the rat's peak  $\text{VO}_2$  response ( $\text{VO}_{2\text{peak}}$ ) produces results similar to those found when the more strictly defined criteria are applied (25, 101). Although the more strictly defined criteria can be used for measuring  $\text{VO}_{2\text{max}}$ , the rat can perform an additional abbreviated maximal exercise test 48 hours after the initial exercise test (147). This abbreviated maximal exercise test is used for two reasons. First, it ensures that a true  $\text{VO}_{2\text{max}}$  is measured for each animal, and second, it minimizes the possibility that the rat reaches the point of fatigue before achieving its true  $\text{VO}_{2\text{max}}$ . An abbreviated maximal exercise test also minimizes the possibility that other

environmental factors could affect the rat's ability to perform on the treadmill (e.g., high ambient temperatures).

One factor that will affect  $\text{VO}_{2\text{max}}$  is the acute instrumentation of the animal. Many investigators acutely instrument rats with cannulas surgically inserted into the carotid artery or the jugular vein. As demonstrated by Flaim and colleagues (103), if the surgery is performed with a short-acting inhalant anesthetic such as halothane, the animal will demonstrate stable cardiac and circulatory hemodynamics along with normal arterial blood gases and acid-base parameters during a 1- to 6-hour recovery period. Moreover, these rats will commonly display behavior that appears to be indistinguishable from that found in noninstrumented counterparts. The acute instrumentation by itself, however, will produce a significant reduction in  $\text{VO}_{2\text{max}}$  (110, 121, 122, 152).

***Measurement of endurance capacity.*** Most tests examining the endurance capacity of rats require the animal to run on a motorized treadmill at a submaximal work rate until it reaches the point of fatigue, which is generally defined as the inability to keep pace with the treadmill (112, 147, 324, 383, 392). However, the animal's running style will change over time, with a gradual lowering of the hind haunches as the rat becomes fatigued. When the rat is unable to keep pace with the treadmill even after repeated application of aversive stimuli, the investigator should remove the animal from the treadmill. Fatigue can be determined by checking whether a rat placed on its back shows a diminished or slowed righting reflex (55, 111, 147, 250).

The endurance capacity of the rat is tightly coupled to the glycogen concentrations found in both the liver and skeletal muscle of the resting animal (55, 61). Furthermore, because liver and skeletal muscle glycogen concentrations fluctuate substantially in a diurnal fashion (55, 61), the time of day at which endurance exercise tests are initiated is extremely important. Food deprivation will significantly reduce both liver and skeletal muscle glycogen concentrations. In fact, 24 hours of fasting will reduce muscle glycogen concentrations by 30–40%, whereas it will nearly deplete the glycogen stores in the liver (61, 385). Therefore, testing in the fed or fasted state will significantly influence the rat's exercise performance.

Chronic instrumentation will also affect the rat's endurance capacity by affecting the animal's liver and diaphragm glycogen stores. In this regard, Moore and colleagues (267) found that surgically instrumenting rats with an

aortic cannula decreased both liver and diaphragm glycogen concentrations. These investigators also found that both liver and diaphragm glycogen concentrations remained depressed until the food intake of each animal returned to presurgical levels. Therefore, the food intake of the rat should be measured both before and after surgery, and endurance exercise tests should not be performed until the food intake returns to presurgical levels.

The last factor that can influence the exercise performance of the rat is the environmental temperature. When rats are required to perform exercise in a hot environment, their endurance capacity is clearly reduced compared with animals performing in either thermoneutral or cold environments (112, 324). This reduction in exercise performance is related to the rat's inability to attain a thermal balance because of significant thermal loads during exercise in the heat (111, 324, 344). Subsequently, rats running in the heat increase both body core and hypothalamic temperatures to the point where they cannot continue to exercise (112, 383). Therefore, the investigator should make sure that all exercise tests are performed in a thermoneutral environment (309). Placement of an electric fan in front of the rat may be used to help the animal dissipate heat, as rats do not use sweating as a thermoregulatory mechanism.

#### **4. Effect on the physiological status of the animal**

Running on a motorized treadmill will increase the metabolic rate of the rat in a quantifiable manner relative to the animal's  $\text{VO}_{2\text{max}}$  and to the external workload being performed (25, 40, 41, 345, 353). Treadmill running imposes potent metabolic stress on the animal, requiring significant increases in oxygen delivery to the working skeletal muscle in both a work intensity- and a time-dependent fashion (9, 11, 220, 221). Chronic exercise training on the treadmill increases the rat's  $\text{VO}_{2\text{max}}$  and leads to adaptations in both the cardiovascular and skeletal muscle systems (19, 25, 51, 65, 84, 101, 122, 159, 281, 392). Furthermore, these training-induced adaptations are associated with large increases in the endurance capacity of the animal (65, 216).

### **B. Exercise Wheel Running in Rats**

#### **1. Rationale for the use of this exercise modality**

Spontaneous wheel running (also known as voluntary wheel running) involves the use of running wheels to chronically exercise rodent species. In



most cases, the running wheels are made of stainless steel and are about 1 m in circumference, although some wheels are smaller and fabricated with plastic (172). This exercise modality has been used successfully in rats, mice, and hamsters, as these animals display an inherent drive to run when given access to activity wheels. One important advantage of using spontaneous wheel running—which is a compelling rationale for its use as an exercise intervention—is that the exercise training can be accomplished with minimal intervention by the investigator. This training modality does not require the use of aversive stimuli (e.g., electric shocks, air jets) to motivate the animals to run in the wheels. Therefore, there is no issue of the appropriate use of aversive stimuli for modification of exercise behavior. Unlike swimming, spontaneous wheel running by normal rats does not induce hypertrophy of the adrenal gland or increase the catecholamine content in the left ventricle of the heart (331), indicating that this modality is not associated with classic indexes of chronic stress responses.

Another compelling reason for using wheel running as an exercise modality is that it offers a long-term mechanism for increasing physical activity in rodents. Rodents can be housed in wheels for many months for investigation of the physiological adaptations that occur in response to exercise training. This is especially important for aging studies (157, 158, 160).

However, spontaneous wheel running does have one critical disadvantage, in that the investigator cannot easily regulate the duration or intensity of the running behavior, except by a dietary intervention (i.e., food restriction) (156, 275). The animal has complete control over the amount and intensity of its running behavior, which can sometimes be erratic. Nevertheless, wheel running has been used very successfully for a variety of physiological and pathophysiological studies of adaptive responses to exercise of cardiovascular, endocrine and metabolic, neuromuscular, neurological, and immunologic parameters.

Spontaneous wheel running is not a good choice for studies that require rats to exercise until they become fatigued or exhausted. The animals rarely run for longer than 2 minutes during each running bout, and they end each exercise bout well before reaching fatigue or exhaustion. However, voluntary wheel running does lead to substantial increases in maximal aerobic capacity in normal (134, 150, 215, 396) and hypertensive (197, 296) rodent models.

## 2. Appropriate use of this modality

**Running patterns.** Rodents, especially rats, in running wheels show typical running patterns, and the investigator must decide whether these activity behaviors are suitable for the scientific hypothesis being tested in a given exercise training study. Once an animal is given access to the activity wheel, there is normally an adaptation period of 2–4 weeks before a plateauing of maximal running activity is reached (134, 149, 150, 155, 265, 320, 323). The rat can maintain this running activity plateau for many weeks, although typically, as the animals age there is a steady decline in running activity (134, 156, 266, 296).

Animals in activity wheels typically distribute themselves among groups achieving high, intermediate, and low overall activity levels (266, 323). For example, young, physiologically normal rats in the high-activity group can average as much as 18–20 km/day for up to 5–6 weeks (150), compared with more typical maximal running activities in the range of 12–14 km/day (155, 323). Intermediate-activity groups display running activities in the range of 6–9 km/day, whereas those in low-activity groups achieve 2–5 km/day (323). Values for minimally acceptable running activities are entirely arbitrary, although a cutoff value of 2 km/day has been used in several studies (150, 265, 323).

**Measuring distance run.** At the very least, investigators using exercise wheels should measure the total daily distance run by each animal by recording the revolutions run in each 24-hour period with an attached revolution counter and then calculating the distance covered based on the wheel's circumference. Because the vast majority of running activity is performed during the dark cycle of the 12:12-hour dark-light cycle (266), investigators should record values from the wheel odometers at the same time each day, usually soon after the end of that day's dark cycle.

**Measuring speed, intensity, and duration of running bouts.** The investigator can assess the speed, intensity, and duration of each individual running bout with external monitoring devices attached to each activity wheel (266, 296, 323). An animal's running behavior over a given 24-hour period typically can be described as intermittent, of high intensity but short duration, and variable. Rats do most of their running during the first few hours of the dark cycle (266, 323). During a typical 24-hour period, immature rats can complete, on average, 100–200 individual running bouts that last 40–90 seconds each, and their running speeds are typically 40–45 m/min (323).

However, slightly faster running speeds (60–70 m/min) have been reported in young female hypertensive rats (296). Animals that display high running activity tend to have more frequent running bouts lasting much longer and typically do not run substantially faster during each exercise bout (323). The typical decline in total running activity observed in most mature and aged rodents relative to younger counterparts is due to reductions in both running speed and duration (266).

Some running wheels are equipped with controllable resistance. When rats have access to wheels with adjustable resistance, the number of revolutions run will decrease at some critical level of resistance. However, the amount and rate of work can be measured by calibrating the resistance to rotation of the wheel. Using higher resistances can induce hypertrophy of leg muscles that do not typically hypertrophy during wheel training without added resistance (172).

**Monitoring.** Spontaneous wheel running differs from other exercise modalities, such as treadmill running or swimming, in that it does not require the continuous physical presence of the investigator to assess activity and ensure safety, and the investigator has little or no influence on the actual running done by the animal. However, the environmental conditions in which the animals are housed must be carefully regulated. For example, the distance run can be affected by the amount of light to which the rat is exposed (dependent on whether the rats are housed on the top or bottom shelf of the animal rack), the activity of the neighboring animals, the estrus cycle of female rats, and many other conditions. The variable response of running activity within a given group of animals must be an important consideration for investigators. Animals placed in activity wheels will typically distribute themselves among groups achieving high, intermediate, and low overall activity levels (266, 323). For example, young (initial age of 5–6 weeks), physiologically normal rats in the high-activity group can average as much as 18–20 km/day for up to 5–6 weeks (150), although maximal running activities in the range of 12–14 km/day are more typical (155, 323). Intermediate-activity groups display running activities in the range of 6–9 km/day, whereas low-activity groups achieve 2–5 km/day (323). Values for minimally acceptable running activities are entirely arbitrary, although a cutoff value of 2 km/day has been used in several studies (150, 265, 323). No reports specifically documenting species-specific differences in running volume or pattern in rats have been published.

***Preventing injuries.*** The only potentially serious adverse consequence of wheel running in rats is that their toenails can break off and they may develop abrasions on their hindpaws during their first few weeks of running. In some cases, an animal may refuse to run until the condition clears up. As with other exercise modalities, such as treadmill running, investigators can prevent potential problems with broken toenails by clipping the toenails or applying a strong adhesive to them. If the injury to the paws lasts for more than a few days, even if running activity is maintained, these animals should be evaluated by a veterinarian and taken out of the study. For long-term exercise training studies, animals should be allowed to recover from the injury for 4–7 days before being reintroduced into the study.

***Consideration of potentially confounding responses.*** A further consideration when using wheel running is that substantial hypertrophy of hindlimb muscles and myocardium typically develops. For example, significant hypertrophy of the soleus and plantaris muscles can be detected within 1–2 weeks of the beginning of wheel running in young rats (149, 150, 274, 321–323). Moreover, the high intensity of the individual running bouts causes significant hypertrophy of the heart (149, 151, 331), a response not typically observed in most endurance-based treadmill running investigations. In some cases, the hypertrophic response can be a confounding variable in the interpretation of adaptive responses in these tissues.

Extreme food restriction can cause deleterious adaptive responses in rats allowed to run freely in activity wheels. For example, restricting access to chow for 1 hour a day during the light cycle can lead to increased running activity, but it can also alter the circadian cycle of gross motor activity, induce a deleterious decline in body temperature during the dark phase, and lead to the development of gastric ulcers (275).

### **3. Factors influencing performance**

***Impact of age.*** Age has a well-documented effect on running behavior. Although spontaneous running can be quite substantial (up to 14–20 km/day) in younger animals (those that started running at 5–6 weeks of age), the amount of running that rats do decreases substantially (by more than 75%) as they mature and reach old age (160, 266). Nevertheless, several studies have shown that mature and older rodents develop positive adaptive responses to enhanced physical activity (128, 156, 207).

## 4. Effect on the physiological status of the animal

**Compromised animals.** Voluntary wheel running has been used successfully for exercise training in different rodent models of hypertension, such as the spontaneously hypertensive rats (296, 331) and the male heterozygous TG(mREN2)27 rat (197). In addition, a limited number of published investigations have used voluntary wheel running in insulin-resistant rodent models (136, 286, 306, 399). However, voluntary wheel running may not be appropriate for animals with massive central obesity (e.g., obese Zucker rat) because anecdotal evidence indicates that they are unlikely to do much spontaneous running (E. J. Henriksen, unpublished observation).

It should be emphasized that spontaneous wheel running is not a modality in which maximal effort leading to fatigue or exhaustion during the running bouts can be achieved. The animals rarely run for longer than 2 min during each individual exercise bout and clearly end each exercise bout well before fatigue or exhaustion is reached. However, voluntary wheel running leads to substantial increases in maximal aerobic capacity, as assessed with an incremental treadmill test, in normal (134, 150, 215, 396) and hypertensive (197, 296) rodent models.

## C. Swimming in Rats

### 1. Rationale for the use of this exercise modality

Most terrestrial animals have the innate ability to swim and are good swimmers when necessary. Because rodents are the species used most widely in experiments involving swimming as an exercise modality, this section focuses primarily on the use of swimming in rats. However, many of the issues discussed below can be applied to other animal species.

Swimming has been used extensively in a wide variety of behavioral and exercise studies, and the classic review by C. A. Dawson and S. M. Horvath (68) should be required reading by anyone contemplating using this exercise modality for rats. Swimming can be used to identify the physiological, biochemical, and molecular responses to acute exercise stress and the adaptations to chronic exercise training (16, 167, 182). Swimming requires less expensive and less elaborate equipment than treadmill running and spontaneous wheel exercise, although the investigator must carefully select the container in which the rats will swim, as well as the temperature and

depth of the water. In addition, swimming can provide a more uniform type of physical activity when performed appropriately (e.g., it does not necessarily involve “stop and go” activity such as that found with treadmill running). Finally, compared to treadmill running and spontaneous wheel exercise, swimming has the advantage of not causing foot injuries, so it may be less physically traumatic to the animal.

One distinct disadvantage of swimming is that some animals will not demonstrate continuous swimming behavior but will resort to diving or bobbing behavior. These responses may be construed as escape or survival strategies that the rat uses to avoid the stressful possibility of drowning. Therefore, these types of behavior can confound the interpretation of results by introducing intermittent bouts of hypoxia. The investigator can minimize these effects by making sure that the water is deep enough to minimize and/or eliminate these types of behavior. However, if the unacceptable diving and bobbing behaviors cannot be eliminated, the investigator must seriously consider removing these animals from further study.

## 2. Appropriate use of this modality

**Water tank design.** The size and the shape of the tank used for the swimming regimen may influence the rat’s exercise performance. Round tanks are a better choice than square tanks, because animals cannot hang in the corners and reduce their swimming intensity. The tank should be deep enough to eliminate bobbing, and the distance from the waterline to the top of the tank should be great enough to prevent the rats from pulling themselves up and out of the water. Finally, the tank should provide a sufficient water surface area for the animals to swim. Water surface areas in the ~1,000–1,500 cm<sup>2</sup> range appear to be appropriate (13, 63, 102, 224, 278, 360). Whether rats swim in tanks with clear or opaque sides does not appear to influence performance. However, if the rats swim to the point of fatigue, opaque sides reduce the variability within and between animals (252).

**Swimming behaviors.** Swimming behaviors of rats are not uniform. They must swim continuously for this exercise modality to be appropriate for applying exercise stress. Other types of swimming behavior—including floating, climbing, diving, and bobbing—are not appropriate for either acute or chronic exercise studies because these behaviors typically induce hypoxia. Therefore, investigators should ensure that rats swim continuously by using the techniques described below. If the rats engage in other swimming

behaviors and these cannot be prevented, investigators must remove these animals from the study to prevent factors other than exercise from confounding the interpretation of the results.

**Continuous swimming.** Continuous swimming involves continuous movement of the rat's forelimbs and hindlimbs while maintaining its snout above the waterline. Both the forepaws and hindpaws remain submerged while the animal exercises, but sometimes its head may submerge slightly for a very short period of time. Continuous swimming can involve mild exercise when rats swim without weights attached to their tails, and it can involve moderate to heavy exercise when weights are attached to their tails (see below). Continuous swimming produces significant recruitment of both the forelimb and hindlimb muscles (127, 175), although the pattern of recruitment may differ from that which occurs with treadmill running, based on glycogen depletion and skeletal muscle blood flow studies (10, 11, 13, 224). Finally, rats do not become hypoxic when they swim continuously (102, 116). Five types of swimming behavior have been described in the literature, including floating, continuous swimming, climbing, escape behavior in the form of diving, and bobbing (17, 74). These different types of swimming behavior are discussed in detail below.

**Floating.** Rats can swim continuously in nonturbulent isothermal water for as long as 60 hours (318). However, whether or not the rats swim continuously is unknown because these animals can float. Rats will create a significant amount of turbulence in the water, and in the process trap air bubbles within their fur, thereby increasing their buoyancy (252). In fact, investigators have noted that some rats can increase their buoyancy so much that they will actually fall asleep as they float in the water. Therefore, one must control the amount of air trapped in the fur to avoid the floating type of behavior.

Several tactics can be used to reduce floating. Some investigators shave the animals before letting them begin swimming. In addition, adding a small amount of detergent to the animal's fur or the water while agitating the water continuously seems to make it impossible for the rat to float (102, 252). Finally, adding weight to the animal's tail (see below) can counteract the increased buoyancy produced by the trapping of bubbles in the animal's fur (252).

**Climbing.** Climbing behavior is very similar to continuous swimming except that the forepaws and forelimbs of the rat break the water's surface in a rhythmic fashion (74). This behavior is part of the "escape behavior" of naive animals (those new to swimming) early on during a swimming bout (74), and it tends to disappear as animals become familiar with swimming or they are trained in this type of exercise. If the rat continues to climb for any length of time during an exercise bout, it is likely to become fatigued quickly. Putting the rats in round tanks with large surface areas and keeping a fair amount of space between the water level and the top of the tank tends to reduce climbing.

**Diving.** Naive swimmers usually dive in an effort to escape from the water. At first, rats try to climb out of the water tank. When this fails, some rats dive under the water to try to find an escape route. Thus diving has been categorized as part of the "escape behavior" that a rat will display during the early part of a swimming regimen. As the rats learn that no escape route is available, they tend to stop diving and climbing. Behavioral scientists at first described this adaptation as "learned helplessness" (32, 318), although more recent studies suggest that the rat learns to reduce both diving and climbing activities to conserve energy (42).

**Bobbing.** Rats swimming in relatively shallow tanks learn to sink to the bottom to rest and push off to return to the water's surface to breathe (201). In fact, some rats begin this strategy within 20 minutes of swimming in a 51-cm-deep water tank (17). This type of swimming behavior, known as bobbing, seems to be a type of survival strategy used to conserve energy. Rats that swim continuously have been found to perform at a rate of 3 metabolic units (METs), whereas rats that bob perform at 2 METs (17). When they bob, rats may spend as much as 60% of their total swimming time submerged (360), and they become hypoxic (360). It is clear that this type of activity should not be considered exercise.

Rats tend to bob when they swim in water that is 50 cm deep or shallower (17, 102, 360). To reduce bobbing, rats should be placed in water tanks that are at least 100 cm deep (252) or weights should be added to the base of their tail (see below) and the water should be constantly agitated (102). Making the water so deep that rats cannot reach the bottom of the tank while holding their breath can eliminate bobbing behavior. Under these circumstances, rats usually swim continuously until fatigue sets in or the exercise session is terminated (252).



**Measuring workload.** As with treadmill running, the workload of the swimming rat can be clearly defined in terms of the animal's maximal heart rate ( $HR_{\max}$ ) response or  $VO_{2\max}$ , if the technology is available. However, it is technically more difficult to measure these parameters in the aquatic environment.  $VO_2$  must be measured only when rats are swimming continuously to accurately reflect the animal's metabolic rate.

**Caring for animals after swimming.** The investigator must make sure that all animals are dried and placed in a warm environment after finishing their exercise bouts. For example, animals can be placed under a heat lamp briefly to dry off.

**Monitoring.** A designated observer must focus completely on the animals during the exercise testing or training period because rats can drown quickly. Each observer must have defined criteria for terminating the exercise session (e.g., length of time submerged). A submersion time of approximately 3 seconds could be set for ending the session as long as the rat is not diving or bobbing. Constant monitoring of the animals must be strictly enforced, as failure to comply may be deemed an inhumane procedure.

### 3. Factors influencing performance

**Environmental factors.** Swimming performance in the rat is substantially influenced by water temperature (17, 22, 42, 69, 70, 378). Accordingly, when the water is much warmer than the rat's core temperature (i.e., 42°C or greater), the animal becomes hyperthermic, its exercise performance diminishes greatly, and death may ensue (22). In contrast, when the water is significantly cooler than the rat's core temperature (i.e., 20°C or lower), the animal becomes hypothermic, its exercise performance is greatly reduced (69), and death may ensue (22). If the water temperature is maintained slightly lower than the animal's core temperature (i.e., between 33 and 36°C), the rat can maintain its core temperature throughout the exercise bout (17, 69). Moreover, rats swimming in water at this temperature range do not experience decrements in various cardiovascular parameters (e.g., cardiac output, heart rate, mean arterial pressure) that could influence exercise performance (69).

**Exercise intensity.** Exercise intensity can vary significantly depending on the type of swimming behavior displayed by the animal. Furthermore, if the rat maintains a continuous swimming behavior, the addition of weight to its body or tail will increase exercise intensity substantially.

Rats learn to trap air in their fur to increase buoyancy. Under these circumstances, the animals can float in the water with very little movement. Oxygen uptake ( $\text{VO}_2$ ) measured in floating rats is very close to that measured during resting conditions on a motorized treadmill. Therefore, the exercise intensity in the floating rat is negligible.

If the rats are prevented from floating and have to swim continuously (without addition of weight to the body or tail), they will perform at a metabolic rate of 2–3 METs with a  $\text{VO}_2$  ranging from 46 to 63  $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}$  body weight<sup>-1</sup> (13, 17, 70, 252). Because the maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) of normal rats ranges from 85 to 100  $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}$  body weight<sup>-1</sup>, this means that nonweighted swimming rats are performing within at a moderate intensity (~45–65% of  $\text{VO}_{2\text{max}}$ ) (13, 252, 278).

To increase the exercise intensity of the swimming rat, investigators may add weight to the animal's body or tail (usually the tail). The amount of weight added is usually based on total body weight (63, 102, 226) and should not be so great as to submerge the animal. Moreover, the amount of weight added must still allow the rat to swim continuously. If, for example, a weight representing 2% of the rat's body weight is attached 2 inches from the end of its tail, the rat's  $\text{VO}_2$  will be ~81  $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  (252), compared to the normal  $\text{VO}_{2\text{max}}$  of rats in the 85–100  $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  range, so this workload would be classified as high intensity. When a weight equal to 4% of the rat's total body weight is attached at the base of the tail, the animal performs within 65–70% of its  $\text{VO}_{2\text{max}}$  (278). Placing the weight closer to the end of the tail may also change the swimming biomechanics and, therefore, the mechanical efficiency at which the rat can perform.

#### **4. Effect on the physiological status of the animal**

Swimming is different from treadmill running or spontaneous wheel exercise in that the form of locomotion and the muscles used produce significantly different muscle recruitment patterns and exercise intensity (72, 88, 328). Although rats use similar stride frequencies when running and swimming, the ankle and leg extensor muscles are more heavily involved during running, whereas the ankle and leg flexor muscles are recruited more heavily during swimming (11, 224). Even though the range of motion in the hip region of the rat is similar during running and swimming, swimming produces a greater range of motion in both the knee and ankle joint regions than running (127). As a result, muscle recruitment patterns may affect the

effort and intensity of exercise performance. These differences between the biomechanics of running and swimming in the rat may also be found in other species.

With treadmill running, the workload that each animal performs can be clearly defined within the context of the animal's maximal heart rate ( $HR_{\max}$ ) response or the animal's  $VO_{2\max}$ , if the technology is available. Similarly, during swimming the workload can be defined within the realm of the animal's  $HR_{\max}$  and/or  $VO_{2\max}$ , although it is more difficult technically to measure these parameters in the aquatic environment.  $VO_2$  must be measured when continuous swimming is displayed in order to accurately reflect the metabolic rate of the animal.

### III. EXERCISE MODALITIES IN MICE

#### A. Background and Introduction

Three primary reasons exist for using mice instead of other, typically larger animals to study exercise. First, the wealth of information available regarding the mouse genome makes it possible to identify the genes involved in acute and chronic exercise adaptation. In December 2002, the complete sequence of the mouse genome was published by the Mouse Genome Sequencing Consortium. This provides an invaluable resource for identifying potential candidate genes involved in exercise performance and/or adaptation. The completion of the human genome sequencing project makes it possible to identify homologous human genes that may be involved in exercise performance and adaptation. In addition, mouse and human gene sequences can be compared to identify homologous regulatory sequences in candidate exercise-responsive genes that may make it possible to identify shared regulatory pathways involved in transducing exercise signals in these species.

Second, because of their high fertility and short gestation, mice can be bred fairly quickly and easily to study the heritability of a particular behavior or trait. A large number of inbred mouse strains have been generated through decades of inbreeding. Many of these strains differ significantly in both voluntary and involuntary running exercise performance (229), which may allow for the future identification of polymorphisms and genes affecting exercise behavior through genetic crossing and polymorphism identification. In addition, mice can be bred for high exercise activity and subsequently studied. These traditional breeding techniques have been used to select for

mice with high voluntary wheel running activity, and several physiological differences between these mice and matched controls have been identified (114, 163, 179, 361, 362).

The third, and most compelling, reason for using mice in exercise studies is the widespread and increasing availability of transgenic approaches to manipulate the mouse genome. The mouse remains the overwhelming species of choice for creating null or transgenic animals in which a single gene is either disrupted via homologous recombination or overexpressed by incorporating multiple copies of the transgene into the genome. The ongoing proliferation of genetically defined mouse models for a wide range of disease states has invigorated the use of mice in the study of physiology and disease. In particular, mice are being used more frequently in models requiring exercise assessment (30). Assessment of exercise phenotypes in inbred or genetically modified mice can include various parameters, such as volitional propensity to exercise, exercise tolerance, endurance, and physical ability to exercise.

Studies on null and transgenic mice allow researchers to identify the role of a specific gene product in exercise performance and adaptation (141, 142), and they account for a growing number of publications related to exercise biology. However, one caveat is that most traditional methods for inactivating or overexpressing a single gene product typically do so in all cells and over the organism's entire life span, so that decrements in exercise ability or adaptation response may not be a direct consequence of the gene in question but rather a secondary consequence of the effects of gene inactivation on some other organ system or during some earlier phase of development. Conversely, the lack of an exercise phenotype may not exclude a role for a particular gene product, because secondary adaptations may compensate for the loss or overexpression of a particular gene (5). Still, null and transgenic technology provides the most straightforward way to test the role of a single gene product on exercise, and newer approaches for creating "conditional" null or transgenic animals—which permit the manipulation of gene expression in specific cells/tissues or at specific time points—will greatly improve our understanding of the contribution of individual gene products to a complex behavior such as exercise.

It should be noted that, in contrast to rats, considerable strain differences exist among mice for performance in treadmill running, wheel running, and swimming. These are specified in relevant sections that follow.

## B. Treadmill Running in Mice

### 1. Rationale for the use of this exercise modality

Treadmill running exercise has one main advantage over voluntary wheel running and swimming exercise—it allows the researcher to precisely control the level of exertion and make it uniform for all the mice in the study. In treadmill protocols, both the duration and the intensity of exercise can be manipulated, and mice can be made to exercise at either submaximal or maximal workloads. In addition, decrements in graded treadmill exercise test performance can be used as a diagnostic indicator of cardiovascular or other defects in mice (99).

The major drawbacks to using treadmill exercise are handling by the researcher and use of aversive stimuli to encourage running. In addition, treadmill training requires a specialized treadmill, which is a more elaborate and expensive apparatus than that required for swimming or wheel running. Finally, treadmill running, like swimming, requires constant vigilance by the researcher to make sure that animals run for the entire exercise bout.

Treadmill exercise performance differs by mouse strain, but no consensus has been reached on which strains perform best. According to some studies, Swiss Webster mice are among the best treadmill runners and C57BL/6J mice are among the worst (229, 231).

### 2. Appropriate use of this modality

**Treadmill design.** Mice can run on a treadmill system built for rats consisting of a standard treadmill to which a series of steel or Plexiglas lanes has been attached to keep each animal in its own lane. However, because the lanes on a rat treadmill may be wide, mice may expend considerable energy in lateral movement. For this reason, a lane system designed specifically for mice should be used; such lanes are typically approximately 4–6 inches wide and 36 inches long. Treadmill speeds should range from less than 5 meters/minute up to 40–50 meters/minute, with small enough increments to allow mice to increase running speed without undue difficulty (typically 5 meters/minute). In addition, the treadmill elevation should be adjustable so that investigators can study the effects of uphill and downhill (eccentric) running.

***Familiarization.*** Like rats, mice require familiarization with the treadmill apparatus to minimize psychological stress, which can interfere with exercise performance or mask exercise adaptations. The familiarization process should start gradually and continue for several days before the actual treadmill exercise running protocol begins. Mice should be placed on the treadmill while it is not operating to become familiar with the sight and smell of the apparatus and the exercise training room. This should be done for 5–15 minutes per day at least once per day for several days. The mouse should then be familiarized with the sounds and experiences of the moving treadmill by turning the treadmill on at the lowest speed setting and allowing the mouse to walk or run slowly for 5–15 minutes. Familiarization sessions longer than 15 minutes or at higher speeds are not recommended because they can induce training adaptations.

Familiarization and training regimens for mice, as well as behavioral definitions of exhaustion, are available in the literature (66, 67). Some strains of inbred or genetically manipulated mice may require modified training schedules of shorter duration, lower intensity, or decreased frequency than wild-type mice.

***Aversive stimuli.*** Although most mice run willingly for short periods (a few minutes) at relatively low speed on a treadmill after familiarization, some type of aversive stimulus is usually required to maintain running behavior for more than a few minutes at low speeds. Three different methods can be used to encourage mice to continue running: tapping their tails or hindquarters lightly with a stick, blowing puffs of compressed air on their hindquarters if they get too close to the back of the treadmill, and placing an electric shock grid at the back of the treadmill to deliver a mild electric shock. The level of shock should not be so high as to produce damage or burns to the animal. Mice that do not exercise willingly after several training sessions or that require extensive prodding or several electrical contacts per session should be excluded from study.

To date, the literature has not included any reports of the use of rewards, such as food, to encourage mice to run on a treadmill. However, given that such systems have been used successfully in rats, they likely could be utilized in mice as well (392).

***Duration and intensity.*** Researchers can manipulate the duration, intensity, and frequency of treadmill training bouts. Duration can range from

a few minutes to a few hours, depending on the outcome variables being studied; most treadmill training regimens use 30- to 120-minute durations per exercise bout (189) to achieve typical endurance exercise adaptations. Intensity can be manipulated by increasing either the treadmill speed or incline. During maximal treadmill exercise tests, both speed and incline are increased in a graded manner across the exercise test until the animal is unable to maintain the workload. These increases typically begin after the first 2–5 minutes, to allow mice to acclimate to the challenges of the new workload, and occur every 2–5 minutes until the animal can no longer maintain running. During chronic treadmill training paradigms, treadmill speed is usually kept constant during each bout but is often increased throughout the training regimen as maximal oxygen uptake improves (189). The frequency of training bouts can range from twice a day to once every other day depending on the desired outcome.

***Evaluating performance.*** In general, three parameters can be used to evaluate treadmill running performance in mice: 1) running duration at a fixed speed—mice are run at a fixed speed until they can no longer stay off the shock grid; 2) running speed on a graded exercise test—the speed of the treadmill is increased every 2–5 minutes until animals can no longer stay off the shock grid, and the maximum speed attainable by a particular animal is recorded; and 3) beam breaks per minute during a run at a fixed speed—a light beam is placed across the back of the treadmill along with a beam break counter. Animals that are unable to maintain the treadmill speed come off the back of the treadmill onto the shock grid and trip the beam break counter.

***Preventing injuries.*** Because treadmill running requires a basic level of motor coordination and weight bearing, any genetic manipulations or natural mutations affecting these systems may adversely affect the mouse's ability to run on the treadmill. Similarly, injuries to the feet or legs can negatively affect treadmill running performance. Mice should be checked for damage to the feet or toes, and animals that have injuries should be treated promptly and monitored for their ability to continue in the protocol.

### **3. Factors influencing performance**

***Running patterns.*** Mice typically display the same “stop and go” pattern of running as rats in that they often slow down and then speed up either voluntarily or in response to an aversive stimulus. Over longer durations of treadmill running, this “stop and go” pattern increases as the animal becomes

unable to maintain the fixed workload. Failure typically occurs when the mouse requires several aversive stimuli per minute to continue running.

***Environmental factors.*** Environmental variables—such as ambient temperature, handling, circadian time, and familiarity with the treadmill—can have a substantial impact on murine cardiovascular parameters that may be differentially apparent during rest vs. exercise (30). Mice should be run in an environment that minimizes excessive heat buildup.

Mice are nocturnal, so treadmill familiarization and training can be done during their dark cycles. This can be accomplished by changing the light-dark cycle so that the housing room is dark during the day. However, unless the specific aim of the study is to investigate circadian rhythms, this reversal of the light-dark cycle may not be necessary. Investigators should also consider whether or not exercising mice during their normal sleep period produces greater stress on animals, interferes with some physiological responses, and/or blunts exercise performance. Other measures can also reduce stress in the animals, including minimizing the number of people in the room during treadmill training, avoiding loud or sudden noises or movements, having the same researchers do the training each day, and doing the training at approximately the same time each day.

***Sex, age, and strain.*** Both sex and age can affect treadmill running performance and adaptation. Female mice show a greater adaptive range in  $\text{VO}_{2\text{max}}$ , ventricular mass, cardiomyocyte size, and skeletal muscle mass than male mice in intensity-controlled treadmill training (189). This suggests that at the same relative level of exercise intensity, female mice experience a greater level of adaptational benefit than males. Similarly, young mice can run longer at a fixed submaximal treadmill speed than old mice (297).

Treadmill exercise performance differs among different mouse strains. However, as yet there is no consensus on which strains perform best. One group reported that FVB/NJ and Swiss Webster mice performed the best in a maximal speed treadmill test and the BALB/cJ, DBA/2J, and C57BL/6J strains performed the worst (229). In contrast, other investigators reported that BALB/cJ mice performed better than any other strain and Swiss Webster mice performed better than C57BL/6J or DBA/2J strains (231). In both cases, Swiss Webster mice were among the best and C57BL/6J mice were among the worst treadmill runners.



Treadmill exercise performance can also be altered in null and transgenic mice (31, 99, 108, 124, 142, 313). However, in some instances, null or transgenic mice show no change in treadmill exercise test tolerance compared to wild-type mice (115, 170), and this is often due to secondary adaptations in other physiological systems that compensate for the primary genetic defect. For example, mice null for the myoglobin gene have normal treadmill exercise tolerance (115), which can be attributed in large part to adaptations in vasculogenesis and muscle metabolism that compensate for the loss in myoglobin (125).

**Instrumentation.** Instrumentation of mice can adversely affect treadmill running performance. In one study, implantation of a heart telemetry device into mice resulted in a 33% decrease in maximal treadmill exercise capacity (30).

**Toe clipping.** Some laboratories clip toes in young mice, either for genotyping or for animal marking and identification. This practice can adversely affect gait in treadmill studies and is therefore not recommended, and some other manner of obtaining DNA and marking animals should be used.

#### 4. Effect on the physiological status of the animal

Treadmill running produces acute homeostatic alterations consistent with the increase in workload. During exercise, heart rate and  $\text{Vo}_{2\text{max}}$  increase linearly as a function of either duration or intensity of work (73). In addition, blood flow to the exercising limbs (248) as well as glycogen and glucose metabolism (85, 164) are increased during exercise. Finally, concentrations of several cytokines increase after 60 minutes of treadmill exercise (59).

Chronic treadmill training results in increased maximal oxygen uptake or  $\text{Vo}_{2\text{max}}$  (189, 289) that enables mice to run at greater speeds (189). Heart weight, particularly ventricular weight, increases after treadmill exercise training (189). Skeletal muscle undergoes adaptations in response to treadmill training associated with greater oxygen and substrate utilization, including enhanced glucose uptake (364), increased fatty acid oxidation, increased mitochondrial enzyme content (334), and a greater muscle capillarization (351). In addition, skeletal muscle mass is also increased as a result of treadmill running (189).

Prolonged treadmill training in mice can affect the joints. Lifelong daily treadmill training increases the severity of osteoarthritis in the knee joints of male C57 mice (218).

A single bout of downhill treadmill running has also been used extensively as a model for eccentric contraction-induced muscle damage. In mice, a single bout of downhill running at a 5–20% downward slope results in elevations in serum creatine kinase and a decrease in specific tetanic force production in the days following an exercise bout (52). Training mice in downhill running protects against the development of these indicators of muscle damage (240).

## **C. Exercise Wheel Running in Mice**

### **1. Rationale for the use of this exercise modality**

The primary rationale for using wheel running exercise in mice is that it allows these animals to exercise when and at the intensity that they choose. Availability of a running wheel may reduce the effects of chronic stress on depression-like signs in mice (352). Because mice run voluntarily in cage wheels, researchers need not be present during wheel running, except to document the wheel running variables once per day. As a result, this modality is less labor intensive than treadmill running or swimming protocols. In addition, cage wheel and activity monitoring devices can be set up relatively inexpensively and will allow many mice to be trained at the same time (219).

Voluntary wheel running exercise can provide a behavioral readout to determine the effects of a drug or toxin on mammalian physiology and behavior. Typically, the time and distance run on a voluntary running wheel are monitored over several days or weeks to determine whether a particular substance has an effect on exercise behavior (81, 366). This approach can also be used to test whether a particular substance has exercise-promoting effects and therefore may be an ergogenic aid (15). Voluntary exercise levels can be used to assess behavioral/functional defects in both naturally occurring mutant mice, such as *mdx* mice (139), and genetically engineered null mice (141, 142, 372, 386). Such studies have demonstrated that distance, time, and speed of voluntary wheel running can all be reduced in mutant and transgenic mice compared to wild-type mice.

Voluntary wheel running exercise can also be used to determine whether exercise ameliorates the disease phenotype and therefore may be useful as a

treatment modality for humans. Voluntary wheel running exercise decreases disease progression in several mouse models of human neuromuscular diseases, including amyotrophic lateral sclerosis, Parkinson disease, and Duchenne muscular dystrophy (87, 143, 199, 368). Also, voluntary wheel running exercise may enhance natural cytotoxicity (241) and decrease tumor metastasis and progression in several models of cancer (57, 60).

The major drawback of voluntary wheel running is that the researcher cannot control the intensity and duration of exercise. Moreover, mice tend to run on wheels at the lower end of submaximal exercise intensities, and certain lines of transgenic mice may not engage in enough voluntary wheel running exercise to produce training adaptations. Also, the voluntary aspect means that motivation to run is a powerful determinant of distance and time run, and changes in exercise performance may reflect changes in the willingness to run rather than some physiological alteration. Finally, because mice run intermittently throughout their active cycle, voluntary wheel running exercise does not lend itself to studies that require precise timing to explore acute postexercise adaptations.

## 2. Appropriate use of this modality

***Exercise wheel design.*** Establishing a voluntary wheel running exercise program for mice involves essentially the same steps as for rats, except that mice may need special equipment because of their smaller size. For example, most studies on wheel running in rats use wheels approximately 1 m in diameter, whereas studies with mice use wheels approximately 30 cm in diameter (6, 141, 142). However, mice may actually prefer larger wheels over smaller mouse-sized wheels (21). Mice can use larger wheels successfully, but if the wheel is too large the energy required to overcome the inertia from the greater wheel weight may discourage them from exercising. Mice also prefer wheels with a plastic mesh flooring rather than metal rods when given a choice (21) but will run considerable distances in a standard painted wire mouse wheel (6, 229).

***Familiarization.*** In general, mice do not need to be formally familiarized with the cage wheel. Simply leaving the wheel in the cage overnight allows the mouse to familiarize itself with the wheel; therefore, a formal familiarization process is not needed. However, it is recommended that investigators allow the animals to familiarize themselves with the wheel for a day or two before they start officially recording performance variables.

**Monitoring performance.** Cage wheels can be attached to a computer to track time, distance, and pattern of running (219), but bicycle computers can be used if only distance, time, and maximum speed information are required (6, 141). In addition, average speed can be calculated from average distance divided by average time spent on the wheel. All voluntary wheel running parameters should be recorded daily at the same time each day.

In general, the voluntary exercise pattern displayed by mice is identical to that of rats. Mice do the preponderance of their running during the dark cycle (206) and typically show an intermittent pattern of running activity on the wheel, jumping on for spurts of running and then jumping off dozens or even hundreds of times throughout the night (D. L. Allen, unpublished observations). In addition, mice tend not to run at a steady speed on the wheel but will run rapidly and then slow down, in alternating fashion. Depending on sex, age, and strain, mice will run between 1 and 10 hours per night and approximately 1–10 km per night on average (6, 229). However, mice tend to distribute themselves into high-, intermediate-, and low-running-activity levels, and occasionally a mouse will choose not to run on the wheel. The incidence of these nonrunners is typically around 10% (D. L. Allen, unpublished observations).

When given access to a running wheel for several weeks, mice display a biphasic pattern of exercise. During the first 2–4 weeks, mean speed and total distance tend to increase while running duration tends to stay the same (6). The increase in mean speed is mostly a function of increased running distance, probably reflects a multitude of variables, including increased running economy and adaptations in the cardiovascular and muscular systems, and in general can be taken as a indicator of endurance adaptation (142, 229). During the second phase of voluntary running, from 4 weeks onward, mean nightly running duration and distance tend to stay the same over prolonged running times.

**Preventing injuries.** Mice can sustain foot injuries with extended running, although these injuries are extremely rare. In addition, because mice spend a great deal of waking time on the wheel, the wheel can become covered in dried urine over time, which can reduce participation in voluntary exercise. Wire wheels should be removed and cleaned regularly with soap and water to remove any accumulated urine.

### 3. Factors influencing performance

**Environmental factors.** As mentioned above, mice tend to do most of their running during their dark cycle. However, after mice become familiarized with the wheel, they tend to jump on it whenever they are startled, even during daylight hours. Loud noises or handling of the cage can provoke this response, presumably because of fear and the desire to flee. Thus, on days when the cage bottoms are replaced by animal facility personnel, mice may show a small upward spike in running behavior. Conversely, deviations in daily or weekly routines can adversely affect voluntary running behavior. For example, accidentally leaving the lights on during the dark cycle can reduce running behavior significantly.

**Sex, age, and strain.** Voluntary exercise behavior in mice is influenced by sex and age. Although it is not clear why, female mice tend to run longer and farther per night, on average, than male mice of the same age and strain (205). In female rats, estrogen appears to be necessary for running behavior, as ovariectomized female rats have blunted voluntary exercise responses (29). Age also affects voluntary exercise behavior in that older mice tend to be less active than young mice (384).

In addition, different mouse strains vary greatly in voluntary wheel running exercise performance (229). DBA/1J mice are poor voluntary wheel runners and run only around 2 hours and 1 kilometer per night on average, whereas the best voluntary wheel runners, Swiss Webster and C57BL/6J mice, run approximately three times longer and six to eight times farther than DBA/1J mice (229). Average speed, although significantly higher in C57BL/6J and Swiss Webster mice than in DBA/1J mice, is only approximately twice as high (229). Finally, both C57BL/6J and Swiss Webster mice also show a greater increase in average running time across a 2-week running period than DBA/1J mice (229).

### 4. Effect on the physiological status of the animal

Mice respond to voluntary wheel running exercise in a manner qualitatively similar to other mammals, including rats, dogs, and humans. Voluntary wheel running exercise results in an increase in  $\dot{V}O_{2\max}$  as measured during a treadmill test (362). Endurance exercise in mice is associated with an increase in heart size that is predominantly a consequence of increased ventricular wall thickness (6). Body mass does not tend to change with

voluntary wheel running compared to the starting body weight (6) but may decrease compared to age-matched sedentary control mice.

Several weeks of voluntary wheel running results in a significant increase in skeletal muscle mitochondrial enzyme expression (6, 141) and a shift in myosin heavy chain (MyHC) expression toward greater MyHC IIa and decreased MyHC IIb isoform expression (6, 141). Like rats, mice tend to show hypertrophy of hindlimb skeletal muscles, particularly the soleus muscle, after voluntary wheel running on an unencumbered cage wheel (6). Running on a wheel to which resistance has been added can also induce hypertrophy of the tibialis anterior muscle (171).

One of the most exciting recent findings is that voluntary wheel running exercise results in changes in the adult mouse brain associated with increased neurogenesis and synaptic plasticity. Studies have shown that voluntary wheel running increases expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (29, 200, 287) and increases cell proliferation and neurogenesis in the dentate gyrus (379). At present, it is not clear whether or not increased BDNF release and neurogenesis are a general consequence of increased physical activity or whether these changes are specific to voluntary exercise alone. However, swimming for 5 minutes actually decreases hippocampal BDNF expression in the rat (332), which suggests that increased physical activity alone is not sufficient to increase BDNF expression.

Many studies have used voluntary wheel running exercise as a behavioral readout to determine the effects of a drug or toxin on mammalian physiology and behavior. Typically, the time and distance run on a voluntary running wheel are monitored over several days or weeks to determine whether a particular substance has an effect on exercise behavior (81, 366). This approach can also be used to test whether a given agent has exercise-promoting effects and therefore may be an ergogenic aid (15). Voluntary exercise levels have been used to assess behavioral/functional defects in both naturally occurring mutant mice, such as *mdx* mice (139), and genetically engineered null mice (141, 142, 372, 386). These studies have demonstrated that distance, time, and speed of voluntary wheel running can all be reduced in mutant and transgenic mice compared to wild-type mice.

Voluntary wheel running exercise has also been used to determine whether exercise ameliorates the disease phenotype and therefore may be useful as a treatment modality for humans suffering the same disease.

Voluntary wheel running exercise decreases disease progression in several mouse models of human neuromuscular diseases, including amyotrophic lateral sclerosis, Parkinson disease, and Duchenne muscular dystrophy (87, 143, 199, 368). Voluntary wheel running exercise also appears to enhance natural cytotoxicity (241) and decrease tumor metastasis and progression in several models of cancer (57, 60).

## D. Swimming in Mice

### 1. Rationale for the use of this exercise modality

One of the major advantages of swimming exercise is that it recruits a large volume of muscle mass and produces extensive adaptations to the cardiovascular system (184). In addition, swimming requires less expensive apparatus than treadmill running, and the duration and load of the exercise can be controlled to a greater extent than with voluntary wheel running. The major disadvantages of swimming training are that the researcher must be extremely vigilant to prevent the animals from drowning and must prevent noncontinuous swimming behaviors (e.g., floating) that can either substantially reduce workload or produce hypoxia (e.g., diving or bobbing). In addition, forced swimming, like any forced exercise, may produce psychological stress (98) that can mask exercise adaptations.

Significant differences in the ability of mice to adapt to a single acute bout of swimming have been documented for several null and transgenic mouse lines (170, 198, 316, 333). In addition, transgenic mice expressing a reporter gene driven by various lengths of the glucose transporter GLUT4 upstream promoter region were swim trained for 8 days to identify exercise training-responsive elements in this gene (373). In all cases, mice appeared to tolerate up to 3 hours of continuous swimming without difficulty and with no differences compared to wild-type mice but showed differences in postexercise adaptation compared to wild-type mice. Special care must be taken to ensure that null and transgenic mice are capable of swim training without drowning or significant changes in swimming behavior (i.e., increased bobbing, diving, or floating).

### 2. Appropriate use of this modality

**Familiarization.** Mice should be familiarized with the swimming tank before initiation of an exercise study. Mice should be placed in the tank and

allowed to swim for a few minutes on 3–5 different days to allow them to acclimate to the handling and stress of the task. Bouts of familiarization longer than 5–10 minutes may produce exercise adaptations.

**Container design.** Studies of swimming in mice have used tank sizes with water depths ranging from 10 cm (365) to 50 cm (285). In general, the depth of the water should be greater than the length of the mouse from nose to tip of tail to prevent mice from diving to the bottom to avoid continuous swimming (143). An additional 10–15 cm should be left from the top of the container to prevent animals from climbing or jumping out (93). Consideration must be given to the width and length of the tank available and whether or not multiple animals are to be exercised in the same tank. However, group swimming of multiple mice in a single container is not recommended, as animals tend to climb on top of one another, increasing the risk of drowning and decreasing continuous swimming. An alternative is to create a set of Plexiglas grids that can be placed at the top of a larger tank so that individual animals can swim in a defined area (93).

**Swimming behavior.** Five types of swimming strategies used by rats have been described: continuous swimming, climbing, diving, bobbing, and floating (17, 74). Continuous swimming is the preferred behavior for studying exercise. Swimming behavior in mice differs from that of rats in two critical ways. First, mice spend most of their time continuously swimming and much less time diving, bobbing, or climbing than rats (93, 184). To minimize floating behavior, a system for producing water bubbling can be used (93). Second, mice tend to use their forelimbs minimally, and most do most of their swimming with their hindlimbs (93, 184). Studies requiring increased forelimb activity are therefore not compatible with a swimming paradigm.

**Measuring performance.** Swimming is usually used as a stimulus to produce endurance exercise adaptations, so performance is not typically measured. Instead, mice are made to swim for a fixed period of time for a series of days or weeks. However, because animals will adapt during the training and will be able to swim for longer periods of time after several training bouts, total duration of swimming can be measured as an indicator of improved performance. Because mice swim by treading water in a limited area, the distance swum is not typically measured.

**Other considerations.** After swimming, care should be taken to ensure that animals are dried with a towel to avoid decreases of body temperature



following exercise. Artificial warming is generally not necessary, although care should be taken to make sure that the animal room temperature is not below normal room temperature, to avoid excess chilling of the animals.

### 3. Factors influencing performance

**Environmental factors.** Water temperature influences swimming behavior in mice. Mice are typically exercised at a water temperature below mouse body temperature (around 36°C), usually between 32 and 36°C (93, 184), but can also be exercised at room temperature (23°C) (285). Swimming at temperatures lower than 23°C may decrease core body temperature and reduce swimming speed in a Morris water maze test (168).

**Sex, age, and strain.** At present, the impact of differences in sex or age on swim performance in mice is not well established. Both male (204) and female (169, 184, 204) mice have been used for swimming studies, as have young and old mice (98, 293, 297), but few published studies have directly compared the swimming exercise ability or adaptations of mice of different sexes or ages. One study reported that old mice swam for shorter times and had less swimming activity than young mice (297).

Several different mouse strains have been studied with swimming exercise training, including C57/B6J (169, 184), Swiss Webster (204), C3H (293), NIH-Black Swiss (170), and BALB/c (98), but neither swimming performance nor adaptation has been directly compared across strains.

Null and transgenic mice may also differ in swimming exercise performance or adaptation. Significant differences in the ability of mice to adapt to a single acute bout of swimming have been documented for several null and transgenic mouse lines (170, 198, 316, 333). In addition, transgenic mice expressing a reporter gene driven by various lengths of the glucose transporter GLUT4 upstream promoter region were swim trained for 8 days to identify exercise training-responsive elements in this gene (373). In all cases, mice appeared to tolerate up to 3 hours of continuous swimming without difficulty and with no differences compared to wild-type mice but showed differences in postexercise adaptation compared to wild-type mice.

**Exercise intensity.** As with any exercise paradigm, the primary variables that can be manipulated to influence the magnitude of the training response are the length of the training regimen and the frequency, duration, and intensity

of each swimming bout. The length of the training regimen can range from days to months, and the correct training regimen length depends on whether the researcher is studying acute or chronic aspects of exercise adaptation.

Animals can be exercised once or multiple times per day and every day per week. Swimming bouts usually last between 30 and 180 minutes, and bout duration is often ramped up over the course of a training regimen as the animal's swimming ability improves. Intensity of the swimming bout increases with the extent to which the mouse swims continuously instead of floating or bobbing and the amount of weight attached to the animal's tail. However, adding weight to the tail to increase the work requirement may also increase the risk of drowning and thus should not be done without clear scientific justification.

#### **4. Effect on the physiological status of the animal**

In general, swimming exercise in mice has the same acute effects as treadmill or wheel running. During all of these forms of exercise, heart rate (198) and oxygen consumption (397) increase and insulin and glucagon secretion rates (186) change consistent with the increased muscle use, energy expenditure, and sympathetic nervous system activity. Like all mammals, mice demonstrate an acute decrease in heart rate—known as diving bradycardia—that begins instantaneously on immersion, lasts for several minutes, and may mask early changes in heart rate due to increased exertion (56). Corticosterone secretion is also increased after a single bout of swimming to exhaustion.

Conducting chronic swim training sessions (several times per week for several weeks) also produces typical endurance adaptations of the cardiovascular and neuromuscular systems. In particular, swimming produces a 14–25% increase in relative heart size (93, 184) and a 20% decrease in heart rate at submaximal workloads (184) after 1 month of training. A swim training regimen optimized for producing maximal cardiac hypertrophy is 90 minutes per day, twice a day, 5 days a week for 4 weeks with no weight attached to the mouse (93). Swim training also results in skeletal muscle adaptations consistent with increased resistance to fatigue, including increased mitochondrial enzyme activity (184), increased lipogenic enzyme expression (169), enhanced muscle capillarization (351), and decreased muscle fiber size.

## **IV. HINDLIMB SUSPENSION AND IMMOBILIZATION**

In addition to the exercise modalities used in rats and mice described above, several investigations have addressed the physiological adaptations induced under conditions of reduced weight bearing in rodents. The hindlimb immobilization model in rats and mice involves the casting of a hindlimb (100). This model elicits the absolute removal of electrical and mechanical activity of the locomotor muscles of the hindlimb. This technique is used to study the mechanisms underlying the loss of skeletal muscle in the immobilized limb. A detailed description of the methodology and solutions to potential problems arising from this procedure is given in Appendix A.

A related but distinct model of reduced weight bearing in rats and mice is the hindlimb suspension model (3, 367). In this model, the hindlimbs of the animal are suspended above the cage floor and the animal can still use its forelimbs for locomotion. Like the hindlimb immobilization model, the hindlimb suspension model is used primarily to investigate the mechanisms associated with the muscle atrophy and growth failure induced by the model. In many ways, the hindlimb suspension model mimics the loss of weight bearing (known as unweighting or unloading) in the microgravity conditions experienced during spaceflight. In the hindlimb immobilization model, isotonic contractions are still possible and dynamic alterations in electrical activity have been observed. In contrast, in the hindlimb suspension model electrical and mechanical activity of the lower leg muscles are immediately reduced to zero. For example, at the onset of hindlimb suspension, electrical activity of the soleus muscle is reduced to very low levels. However, with more prolonged soleus unweighting, electrical activity in this muscle gradually returns to weight-bearing control levels (4). A comprehensive reference paper on the hindlimb suspension technique intended for researchers, manuscript reviewers, and IACUCs has recently been published (272). A summary of this review is provided in Appendix A.

## **V. SPINAL CORD ISOLATION AS A MODEL OF NEUROMUSCULAR INACTIVITY**

A chronic model of near neuromuscular inactivity has been developed in the rat to address issues related to activity-dependent and -independent influences on skeletal muscle properties (126, 131, 132, 329). This model involves a complete spinal cord transection at a midthoracic and a high sacral

level and bilateral deafferentation (dorsal rhizotomy) between the two transection sites. These animals can be kept in good health for prolonged periods (months), although careful daily care of bladder and bowel function is essential.

With this model of inactivity, the motoneuron-muscle connectivity is intact and, therefore, activity-independent influences, such as neurotrophic factors, are maintained. From an exercise viewpoint, this model has some important advantages scientifically. For example, the model provides for a baseline measure of near-zero activity and allows for a known (quantifiable) amount and/or pattern of activation/loading to be imposed on the muscle via electrical stimulation of the peripheral nerve or spinal cord.

# 4 EXERCISE PROTOCOLS USING LARGE ANIMALS

## I. OVERVIEW

This chapter describes treadmill exercise protocols in large animals—specifically horses, pigs, and dogs. Discussions will focus on treadmill exercise because it is the primary modality utilized by investigators in exercise studies involving these species. For each species, the rationale for using treadmill running is discussed, along with appropriate use of treadmill running, factors that influence study outcomes, expected impact of treadmill running on the animal's physiological status, and concerns related to the use of treadmill exercise in the species.

## II. TREADMILL EXERCISE IN HORSES

### A. Rationale for the use of treadmill running in horses

Exercise studies on horses have dramatically increased our knowledge of equine physiology and pathophysiology. Indeed, evaluation of equine cardiopulmonary function and disease detection at rest and during exercise are now possible at a level close to that in humans. This has benefited horses, through improvements in their treatment and welfare, as well as industries focused on the equid.

The horse is a superlative model of cardiovascular and oxidative function. During peak running speeds, the elite thoroughbred horse can achieve oxygen uptakes in excess of 70 L/min, which is greater than in any other animal. The horse also provides a model of lung failure, with blood gases in the galloping horse resembling those in patients with severe lung disease. Moreover, during high-speed running, the horse experiences exercise-induced pulmonary hemorrhage (EIPH), which is characterized by rupture of the pulmonary capillaries, escape of red blood cells into the alveolar spaces and airways, and, in the extreme, frank epistaxis (91, 92). In addition, the horse becomes severely hypoxemic and hypercapnic while running at high speeds (193, 194, 253–255). Horses are natural high-performance runners and, provided they

have no negative associations with the treadmill, can become accustomed to performing exercise tests at sufficiently high speeds to reproducibly achieve their maximal heart rates and oxygen uptake levels.

## **B. Appropriate use of treadmill running in horses**

**Familiarization.** Thoroughbred racehorses can successfully learn to perform a standardized treadmill test consisting of walk, trot, canter, and gallop between their first and third visits to the treadmill, but they do not appear to be comfortable or particularly stable. Therefore, before actual testing on the treadmill, horses should be familiarized with the treadmill and other equipment such as face masks. Food rewards (e.g., alfalfa pellets) given in a mock-up of the face mask after a treadmill run help the animals form a positive association with the equipment. At least two to four sessions on the treadmill before actual data collection will help ensure that the horses run comfortably and exhibit a stable locomotory pattern. However, up to 6 weeks of familiarization (2–4 times per week) may be necessary for assessing true “resting” responses for horses standing on the treadmill (i.e., heart rate ~30 beats/min) (244).

**Types of exercise protocols.** Three types of running activities are routinely used in horse treadmill exercise studies: incremental running, constant speed, and intermittent running. The format selected should be based on the research or clinical question addressed.

For the extremely popular incremental exercise test to measure maximal oxygen uptake on the flat, the horse typically walks or trots at 3 m/s for 800 m (~4–5 min) before moving to the canter at 7 m/s. At this point, the treadmill speed is increased by  $1 \text{ m}\cdot\text{s}^{-1}\cdot\text{min}^{-1}$  until the horse fatigues. Today, many modern treadmills can achieve 16–18 m/s, which is sufficient to reach maximal oxygen uptake in almost all horses. Because horses have a slow component of the oxygen uptake response (217), even the fastest and fittest horse will eventually reach maximal oxygen uptake before fatigue at these speeds. This occurs even if the top treadmill speed is below the horse’s peak achievable speed but that speed is sustained until fatigue (307, 308). On an incremental exercise test, most horses peak at 15–17 m/s on the flat, and this peak speed is reduced considerably on the incline (10–12 m/s at 10%).

In constant-speed protocols, the horse transitions between rest or a low speed and a higher speed. Just as horses accelerate rapidly out of the gate at the racetrack, modern treadmills bring the horse up to the desired speed within

5–10 s (117, 217). A typical protocol for a constant-speed test might include a warm-up consisting of walking or trotting for 1–5 minutes, followed by a slow canter at 7 m/s or a gallop at 13 m/s for 5 or 6 minutes or until fatigue, followed by a walk or a trot for a cooldown.

Like constant-speed protocols, intermittent running protocols involve a transition between rest or a low speed and a higher speed. Intermittent running may involve repeated bouts at the same speed or progressively increasing speeds interspersed with several minutes of recovery at rest or walking. Intermittent exercise protocols can induce more extreme physiological and metabolic responses (e.g., blood lactate and body temperature increases) than incremental or constant-speed tests performed to fatigue.

Other test protocols are designed to replicate an activity or competition in the field. One example of this is 3-day eventing, where different environmental conditions may be established on consecutive days and a constant-speed endurance test is designed.

**Cool down.** After a maximal exercise test or any high-speed running, it is crucial to cool the horse down properly. This can be accomplished with several minutes on the treadmill at 3 m/s, followed by 20–30 minutes of hand-walking on grass after each test until resting heart rate returns to baseline. In addition, the horse may be given a cool bath (legs first) and allowed to consume moderate amounts of water.

**Treadmill incline.** The treadmill's rubber belt is firmer (disadvantageous) and more even (advantageous) than most other surfaces on which horses usually walk or run. To reduce the concussive impact of treadmill running on the horse's forelimbs, the treadmill can be inclined. A treadmill incline of 3.5% produces a heart rate response that is indistinguishable from that in a horse with a rider running on the flat. However, inclined treadmills yield higher values than flat surfaces for maximal cardiac output and oxygen uptake (254, 255), exacerbate EIPH (196), and increase strain on the hindlimbs (363). Continuously trotting horses uphill on a 5% incline at 5 m/s can make them lame (244). However, horses can run up an incline that is as much as 12%.

Incremental exercise tests performed on an inclined treadmill (up to 10 or 12%) may be more problematic from a design perspective. Specifically, to identify a gas exchange or lactate threshold, data are often collected at

several subthreshold speeds (253), which for the inclined but usually not the level treadmill requires that data be collected at speeds of 3–7 m/s. These speeds often cause the horse to adopt a choppy gait, which disrupts the smooth progression of cardiorespiratory and metabolic variables at these lower speeds.

***Preventing injuries.*** Any form of exercise carries the risk of orthopaedic injury such as bowed tendons. If this occurs on the treadmill, exercise should be halted immediately and appropriate veterinary treatment given. Lameness can develop in any exercising horse, and affected animals should be rested. Under veterinary supervision, appropriate diagnostic procedures and therapy should be performed. Other exercise-related injuries (e.g., exertional rhabdomyolysis) are rare; diligent clinical monitoring will ensure that if these injuries do occur, they will be detected early. Any exercise bout should be terminated immediately if adverse effects become significant (e.g., ataxia), and treatment should be initiated. Exercising animals under analgesics is not recommended as it can lead to catastrophic injury.

Investigators are strongly advised to equip horses with an overhead safety harness (surcingle) attached to an emergency treadmill shut-off switch. This apparatus can support the horse's weight and reduces the risk of injury to both the horse and the supervisory staff if the horse stumbles.

### **C. Factors influencing performance**

***Potential concerns regarding different environmental conditions.*** Thoroughbred horses have only 40% of the body surface area per unit mass of humans—in heavier breeds, this percentage may be even lower. In cold climates, such low body surface ratios may be an advantage; however, in normothermic or hot environments, the low body surface ratios pose a great challenge to the animal's thermoregulatory mechanisms. At maximal aerobic capacity, each kilogram of body mass consumes, on average,  $180 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  of oxygen for a fit racehorse and less for other heavy breeds. This is three times the rate of a healthy club-level human distance runner. Consequently, per unit surface area, the horse needs to lose 7.5 times as much heat as the human.

Exercising horses experience a rapid and dramatic rise in core temperature to values of approximately  $43^\circ\text{C}$  or higher (193). Horses can achieve a maximal sustainable sweating rate of 15 L/h, or about  $3 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ , which is about three times higher than in humans. At maximal exercise



levels, horses can store sufficient heat to raise their body temperatures by 1–1.5°C per minute and thoroughbred horses racing on flat surfaces may experience temperature increases of as much as 3°C (244, 245, 261, 262).

The likelihood that a horse will experience severe hyperthermia (i.e., a core body temperature in excess of 42–43°C) (244, 261, 262) is greatest when the ambient temperature exceeds 25°C and ambient humidity reaches 70–90%. Horses that are overweight or unconditioned, or that possess a long coat, are at greater risk of hyperthermia. Equine athletes are often at greater risk of hyperthermia when placed in hot environments without acclimatization, as are horses that have not been given sufficient opportunity to rehydrate after a previous exercise bout. Monitoring the return to preexercise body mass can provide a good indication that adequate rehydration has occurred. In human athletes, a 1% loss of body weight in the form of fluids reduces performance by up to 10%, but the relationship between fluid loss and performance in horses is not known. The horse possesses a large hindgut with 30–40 L of fluid that may be drawn on during exercise and replaced afterwards. The diuretic Lasix (Salix or furosemide) causes a loss of up to 20 kg of fluid and is often given to horses to relieve racing-induced EIPH and improve performance while lowering pulmonary artery pressure (195).

When horses run at high speeds with inadequate airflow, they can develop marked hyperthermia within 1–2 minutes (244). Investigators should also be aware that environmental factors, horse condition, and the adequacy of prior hydration in exercise and other activities can affect the horse's ability to thermoregulate and thus influence the rate of increase in body temperature. This is particularly evident when a drug that impairs sweating (such as L-NAME) is given to the horse (193).

Horses usually stop running when they become too hyperthermic. This threshold may differ among horses but is generally in excess of 42–43°C. Tests at maximal running speed may cause body temperatures to spike up to 43°C, and horses can tolerate this without injury. IACUCs that do not have a clinician who is familiar with the exercise response in the horse should be advised accordingly. However, horses exercising for prolonged periods of time (e.g., longer than 20 min at  $>70\% \text{VO}_{2\text{max}}$ ) that sustain body temperatures of 41°C or greater should be monitored carefully for signs of ataxia or cessation of normal sweating. It is particularly important that study personnel use fans at least 50 cm in diameter to move air over the horse at a speed at least equal to that of the horse.

***Limb care.*** Protective tendon boots (extending past the fetlock) on at least the forelimbs and bell boots (to cover heel bulbs) on at least the hindlimbs should be used during exercise protocols.

***Food intake.*** Unless other dietary considerations are paramount, food should be withheld for 2–3 hours before testing and for approximately 2 hours after testing.

#### **D. Effects on physiological status**

***Definition of fatigue.*** It is just as difficult and subjective to judge the point of fatigue for horses as for humans. Several definitions of fatigue in horses have been used, such as the first time that the horse drops back more than 1 m from the front bar of the treadmill or when the horse has dropped back but has been encouraged humanely back to the front bar two or three times. Fatigue is sometimes defined based on the time point at which the horse cannot be encouraged back to the front bar of the treadmill. A good indicator that the horse is approaching the point of fatigue is that it begins to alternate leads frequently.

When horses appear to be fatigued, study personnel can encourage them verbally or with a riding crop. Some laboratories use a fly whisk or the hand to pat the gluteal area. Usually, two or three light flicks with the whisk or hand are sufficient to encourage the horse to give one last effort. Once the horse begins to slow down, the test should be terminated and cooldown should begin with a trot or walk. The horse should never be struck with spurs, other sharp or damaging objects, or electrical prods. Such mistreatment is unethical and is likely to prove counterproductive, making the animal skittish and unsuitable for further treadmill work.

***Chronic/repeated exercise.*** Because of EIPH and other health-related issues such as soft tissue damage and/or lameness, horses should not be run to exhaustion repeatedly within too short a space of time. In general, maximal exercise tests should not be performed more than once per week or more than eight times in any 3-month period. About 1 week is required for lung lavage red blood cell counts to return to control levels (258). A typical training/conditioning protocol for laboratory horses might include short runs to 70–80% of their peak treadmill speed 2 or 3 days per week. Nasal strips and Lasix both reduce EIPH in controlled laboratory trials (195), and one of these treatments should be considered for horses that develop heavy EIPH unless the treatment interferes directly with the experimental analysis.

### III. TREADMILL EXERCISE IN PIGS

#### A. Rationale for the use of treadmill running in pigs

Several important similarities between swine and humans highlight the advantages of using swine to study responses and adaptations to exercise. Such similarities include:

- Metabolism of foodstuffs (78, 79, 180, 302) and lipoprotein distribution in pigs are both similar to those in humans (54, 79, 242). Therefore, pigs are used in obesity and adipose research (246, 257, 301).
- Pigs are sexually mature early in their long life span (374).
- Despite the common misconception that the larger size of even miniature swine (~30–80 kg) limits their usefulness (185), most swine are relatively docile and can be handled and restrained in low-stress devices (294, 298). In addition, the size of pigs makes it possible to sample large volumes of blood (300–500 ml) for studies of lipids, coagulation factors, platelets, and other factors that are not possible in smaller animals (347).
- Skeletal muscle and cardiorespiratory responses to exercise are similar to those observed in humans, establishing the pig as a good model for studies of the effects of exercise on the cardiovascular system (7, 8, 222).
- The size of the coronary arteries of pigs makes possible trials of percutaneous catheter interventions for revascularization with devices identical to those used in humans (113).
- The pig's cardiovascular system, especially the coronary circulation, is similar to that of humans in the propensity for few native coronary collateral arteries (340, 387) and pharmacology of coronary artery reactivity (96).
- Heart rate and thus metabolic demand on the heart and cyclic changes in coronary blood flow are similar to those in humans (133, 387).
- Atherosclerotic lesions are morphologically similar to those in humans (28, 161, 301). When fed low-fat diets swine develop modest atherosclerosis, but on high-fat diets they develop the full complement of atherosclerotic lesions (28, 228).

Thus, because of their similarity to humans in a variety of physiological realms, pigs are an excellent choice of animal model for exercise studies.

The pig is a suitable animal model for studies involving moderate-intensity exercise (e.g., where the animals achieve 50–75% of maximum heart rate) because this species can exhibit a variety of physiologically relevant acute responses and chronic adaptations. Moreover, in some studies, pigs have performed treadmill exercise at intensities greater than those that produce maximal oxygen consumption (7, 8, 222).

Swine are also useful models for studying a variety of cellular, molecular, and integrative physiological mechanisms of exercise. They can provide fundamental knowledge regarding several physiological systems and a basis for therapies, including targets of pharmacotherapy, for diseases. Swine have a natural tendency toward sedentary behavior (82), so behavioral modifications are required to elicit running behavior in these animals.

## **B. Appropriate use of treadmill running in pigs**

Virtually all exercise studies in the pig involve running on a motor-driven treadmill. To date, exercise studies emphasizing resistance and strength training, flexibility, and motor skills have not been conducted in pigs. It is possible, however, that future studies of the muscle and skeletal strength that result from exercise will use pigs.

This species is regulated by the U.S. Department of Agriculture, so conservative use and more thorough documentation is required.

***Treadmill design.*** The treadmill should typically be 1.5 times the length of the pig to allow some forward and backward movement. Treadmills used for humans will suffice if an opaque enclosure is placed on the treadmill to contain the pig. The other main consideration is avoiding slippage on the treadmill due to the presence of urine, feces, or water.

***Familiarization with treadmill running.*** Exercise is typically started in miniature swine (Yucatan, Sinclair, Ossabaw) when the animals are 6–8 months old, are sexually mature, and weigh 30–60 kg. No studies have been conducted on substantially younger pigs, but such studies are likely to be feasible. Domestic swine can also be used for treadmill running, but domestic breeds can weigh in excess of 100–150 kg at 6 months of age, making these animals too large for most commercial treadmills.

Even before the pigs are familiarized with the treadmill, they should become reasonably familiar with human contact, which requires that research

staff spend time in the pig's cage every day and provide physical contact, including scratching the animal and placing their hands on the pig's chest to simulate placement of a heart rate monitor. During these socialization sessions, researchers can measure resting heart rates with a stethoscope or a telemetry device. Although simple contact with humans can increase the pig's heart rate, making it difficult to obtain a true "resting" heart rate, auscultation with a stethoscope is a practical method and can be used to identify exercise training-induced bradycardia (37).

Pigs should be familiarized with the treadmill and with running over 5–10 sessions during 1–2 weeks with standard principles of animal behavior modification (64). Some socialized pigs can be led onto the treadmill by mild application of pressure in the desired direction or through positive reinforcement with a food reward or fruit juice (37). The first familiarization sessions may simply involve placing the pig on a stationary treadmill and feeding the animal if it demonstrates anxiety toward the treadmill through vocalization, attempts to escape, or excessive heart rate (i.e., >50% of maximum, ~138 beats/min).

Before turning on the treadmill, the technician should make sure that the grade and speed are at low settings to avoid startling the pig. During the familiarization period, exercise time, treadmill grade, and treadmill speed should be increased incrementally to reach a workload that elicits the desired heart rate that for optimal training effects. For example, in week 1 of the familiarization phase, the pigs might walk on the treadmill 4 days a week for just 10 min/day at 0–3% grade and a slow walking speed of ~2.5 km/h; this elicits a typical heart rate of 35–40% of maximum heart rate (compared to the pig's resting heart rate of 20–30% of maximum). During week 2, the pig might walk 20 min/day at a 5% grade and a walking speed of ~3.3 km/h; this will elicit 45–55% of maximum heart rate.

***Aversive stimuli and rewards.*** Aversive stimuli should be avoided unless absolutely essential (2). When a pig completes the session, it should be rewarded for compliant exercise behavior as soon as possible with a food treat or approximately 100 ml of fruit juice and daily feeding. Delaying the reward will result in minimal association between positive reinforcement and the exercise behavior, so this will not effectively shape the behavior.

***Measuring heart rate.*** Heart rate can be measured in a relatively straightforward manner in this large animal and can provide an objective

measure of exercise intensity. Because of the pig's size, its heart rate can be measured with a standard stethoscope (37). Monitoring heart rate is helpful even though exercise at specific grades and speeds of the treadmill has been shown to elicit specific heart rates (33, 37). Individual variability of heart rate in pigs and certain factors such as thermal stress or disease can have profound effects on the relative intensity of the exercise regimen. For this reason, a "one size fits all" approach is not always appropriate in pigs.

**Measuring efficacy.** The efficacy (i.e., the degree of increase in physical work capacity or some underlying biochemical correlate) of the chronic exercise training regimen must be addressed to demonstrate the study's scientific validity. Measures such as increased citrate synthase and other skeletal muscle oxidative enzyme activities have long been considered indications of beneficial exercise training adaptation (159). Alternatively, measuring efficacy by fitness or "stress" tests in conscious pigs can potentially threaten the animal's welfare. Stress tests involving runs to exhaustion have been used in swine (38). However, submaximal heart rate can be used as a valid test. This guideline complies with the Animal Welfare Act in that it involves a method that lessens or eliminates pain and/or distress and therefore enhances animal well-being. Other noninvasive measures in conscious pigs, such as decreased resting heart rate (bradycardia), may be used to assess exercise training efficacy.

Given the validity of tests of exercise efficacy using submaximal heart rates in humans and pigs, submaximal fitness tests may be preferred over maximal fitness tests if the study's aim is to demonstrate the impact of a training regimen on health rather than to assess elite athletic performance.

**Record keeping.** Because the level of exercise effort is, in principle, similar to a drug dose, daily records of exercise bouts must be kept and made available for IACUC inspection. Exercise log sheets should record heart rate and general condition during resting, warm-up, training, and warm-down stages for the familiarization and training phases.

**Ending exercise bouts.** Humane points for ending exercise bouts in pigs should be objectively and quantitatively defined to ensure scientific validity and the animals' welfare. It is recommended that if any of the following responses occur, technicians should decrease the exercise intensity and, if that fails to correct the situation, remove the pig from the treadmill and perhaps the entire study:

- **Excessive heart rate**—If the pig has reached the target heart rate and tolerated this intensity well during past exercise, then a heart rate that is 10% higher could indicate impairment in the form of illness or orthopaedic problems.
- **Abnormal gait**—Pigs rarely have difficulty ambulating on the treadmill, so a stagger or limp indicates that exercise intensity should be decreased.
- **Labored breathing**—If a pig shows labored breathing or is wheezing or rasping, the technician should auscultate the chest with a stethoscope during a heart rate check to determine whether the pig is experiencing pulmonary congestion.
- **Excessive electric shock**—If excessive electric shock (e.g., more than 4 times in 1 minute) is required to elicit compliance, the pig should be considered noncompliant.
- **Falls on the treadmill**—Any fall on the treadmill requires that the treadmill be turned off immediately. Falls rarely happen because of poor coordination, but they may occur if the treadmill has become slippery because of urination and defecation or from water sprayed to cool the pig. If the animal's heart rate, use of electric shock, gait, and breathing are monitored as recommended, then no pig should be so fatigued that it cannot right itself after it falls down.

### C. Factors influencing performance

**Environmental factors.** Pigs do not sweat, so they must be cooled by directing fans at their backs and applying water mist to the head, ears, and back. However, when misting the animals, technicians must take care to avoid dampening the treadmill belt surface, as this can cause slippage and injury to the pig. Body temperature may be monitored with a standard rectal probe if the ambient temperature and humidity are excessive. Routine monitoring of core temperature may be needed for obese pigs, as obesity decreases thermal dissipation.

**Exercise intensity.** Pigs should warm up before achieving the desired level of exercise. During the warm-up phase, pigs should walk at 2.5 and 4 km/h. Treadmill speed and grade should then be increased to elicit a training (“target”) heart rate. Treadmill speed and grade can be combined in different ways to safely produce the same result—a 65–75% maximum heart rate can be achieved by walking the pig at 5 km/h at a variable 5–15% grade (37) or

through 5–13 km/h variable speeds at 0% grade (38). After some exercise, the treadmill should be stopped and the pig should be rewarded with fruit juice or water. At this time, the heart rate should be measured and recorded.

#### **D. Effects on physiological status**

A plethora of literature has established the firm linear relationship between heart rate and oxygen consumption in humans (14), and this has been confirmed in pigs (33, 268). Thus exercise intensity for studies in pigs should be based on a percentage of the animal's maximal heart rate of 275 beats/min (33). Healthy pigs show very little variability in maximal heart rate (33), so it is not necessary to determine maximal heart rate directly in each pig. The desired exercise heart rate to elicit cardiovascular adaptations (health benefits) should be specified as a range (known as the “target zone”) that ensures appropriate intensity and safety for the animals.

Pigs have been trained in numerous studies at between 65% and 85% of maximum heart rate (180–234 beats/min) for 30–75 minutes with almost no major complications (37, 223, 388). Pigs can tolerate 5 days of exercise per week very well, but 4 days/week may suffice (37). The exercise effort suggested here is safe for pigs and effective for eliciting cardiovascular training adaptations. In some cases, depending on the goals of the study, lower levels of effort may be more scientifically appropriate.

Autonomic neuropathy may confound the relationship between heart rate and oxygen consumption, so other end points must also be monitored closely in animals with diabetes. The peripheral vascular disease and resulting orthopaedic problems associated with diabetes can be managed by reducing treadmill speeds and grade (37) to provide an appropriate workload that will elicit the same exercise training-induced cardiovascular adaptations as in healthy pigs (38, 49, 225). If diabetic and obese pigs demonstrate resting bradycardia, increased skeletal muscle oxidative enzyme activity, decreased heart rate during submaximal exercise, and increased physical work capacity (37, 393), the exercise training regimen is effective.

Reduced workloads can be used to adapt pigs with a fully occluded coronary conduit artery to exercise (86, 146, 387). However, the use of pigs with coronary ischemia in exercise studies requires diligent attention to exercise intensity.



Pigs may experience mild skin abrasions on the rump if they try to rest by leaning on the back of the treadmill or on the hind feet from rubbing on the treadmill's rear door. The abrasions should be treated with topical ointments.

## IV. TREADMILL EXERCISE IN DOGS

### A. Rationale for the use of treadmill running in dogs

Dogs have long been used for exercise studies. Dogs offer several of the same advantages as pigs, including relative ease of training and handling and an appropriate size for surgical implantation of catheters, blood flow transducers, electrodes, and other devices. Dogs also offer some of the same benefits as horses; for example, the dog is a superlative model of cardiovascular and oxidative function. Untrained dogs have a maximal oxygen consumption of  $114 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  (280), which is roughly three times higher than that of untrained humans, and trained dogs can have a maximal oxygen consumption of  $150 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  (280), which is over twofold higher than elite cross-country skiers (44).

Although the dog has long been used as an experimental model to provide basic information about physiological function, it is different from humans in important ways. Dogs have proportionally larger hearts and ventilatory capacities than humans, as well as larger spleens that serve as acute sources for red blood cells to increase arterial oxygen content during exercise (234, 380). Dogs generally have a greater proportion of oxidative fiber types in their skeletal muscle and a lower accumulation of blood lactate at the same submaximal workload (280) than humans (44). Dogs have a resting heart rate similar to that of humans but a far higher maximal heart rate ( $\sim 300$  vs. 200 beats/min), and their hearts have a better-developed coronary collateral system. Dog adrenal glands release an equal mix of epinephrine and norepinephrine, whereas human adrenals release primarily epinephrine. The thermoregulatory responses of dogs maintain brain temperature while allowing body temperature to rise to much higher levels than the responses of humans (211). Finally, dogs show much smaller compensatory vasoconstriction of inactive organs during physical exertion (230, 277, 335, 377) than humans (327).

Healthy dogs have been used for exercise studies of virtually every organ system and physiological process. Dogs can also be used in exercise

studies involving disease models. Examples include heart failure (135, 288), coronary heart disease (39), hypertension (230), obesity (264), diabetes (58, 212, 375), and pulmonary limitations (20, 165).

Some dog breeds are more adaptable to the laboratory environment than others. Dogs are often acquired from USDA Class B licensed vendors, who may in turn acquire the animals from local government pounds (when such acquisition is permitted by law), and detailed backgrounds may not be available. However, for some investigators a Class A vendor may be a more preferable source of research subjects (e.g., homogeneity of the breed, known health history).

When choosing a dog breed for an exercise study, an important practical consideration is the temperament of members of that breed. Pit bulls, rottweilers, and Doberman pinschers, for example, may not be suitable for exercise studies because they tend to demonstrate aggressive behavior. For this reason, some laboratory animal research facilities do not permit the use of pit bulls or animals of pit bull lineage in research. Mixed-breed dogs or purpose-bred dogs of specific breeds (e.g., foxhounds) are often better choices for studies involving exercise (279). Mixed-breed animals from Class B vendors are often easier to use and train than their purpose-bred counterparts because they usually have had much more human contact. Investigators should establish a close rapport with dog vendors to ensure that the animals delivered have the proper temperament for exercise research. Research staff should always be careful when initially handling an animal until its temperament is known. A questionnaire is available that may be helpful in predicting a dog's likelihood of aggressive behavior (166).

## **B. Appropriate use of treadmill running in dogs**

***Treadmill design.*** Treadmills intended for human exercise are suitable for use with dogs, although treadmills built especially for dogs may also be used (356). Treadmills used with dogs must be able to operate at a very low speed ( $\leq 1$  km/h) and/or to be moved manually to facilitate familiarization of the animal with treadmill locomotion. A cage purchased from a vendor or constructed from materials available at building supply stores should be placed around the treadmill to keep the animal properly aligned on the belt. The belt should be constructed from or covered with a soft rubber or rubberlike material to provide adequate traction. The harder, more plasticlike belts

found on most human treadmills are adequate for studies involving dogs and are reasonably easy to wipe clean.

**Familiarization.** Optimally, dogs should be thoroughly familiarized with laboratory personnel and the general laboratory environment before they begin to become familiar with the treadmill. While the animal is in the laboratory, the treadmill should be switched on and off to acclimate the animal to the treadmill's noises. Because some animals are startled by loud treadmills and display trepidation toward the machine, the treadmill should be as quiet and as low to the ground as possible. Once the animals have become accustomed to the treadmill's noise, they should be placed on the treadmill belt while the machine is not running to let them become familiar with the machine's sights and smells.

The first few times that the dog is placed on a moving treadmill, the operator should be prepared in case the animal tries to jump over, crawl under, or move through any restraining devices or cage. Initially, the treadmill should be started at the lowest possible speed (0.1 km/h—treadmill barely moving), which greatly facilitates familiarization with the treadmill. To achieve such a slow speed, the technician at the front of the treadmill should advance the belt manually with his or her feet. For the first session, only a walking speed (~2–4 km/h) should be attempted. Training will be facilitated if the animal is walking freely before the treadmill is shut off. Technicians should offer copious praise and food treats during training. Electric shock is unnecessary for training dogs to run on a treadmill because they typically respond favorably to positive reinforcement.

**Monitoring.** Investigators should always stay at the front of the treadmill, holding a leash attached to a harness on the animal. They can pull on the leash when needed for easy, rapid control of the animal if it stumbles or tries to escape from the treadmill. Investigators should also provide verbal encouragement to dogs while they run on the treadmill. Some animals splay their legs and slide on the treadmill. Repeated splaying of the legs after two or three daily sessions, although relatively rare (if the animal is placed on a quiet, highly adjustable treadmill), strongly indicates a poor likelihood of successful training in a short period of time. If this occurs, serious consideration should be given to removing the dog from the study.

**Ending exercise bouts.** When a dog becomes unsure of its footing, it often tries to dig into the belt with its toenails, decreasing the traction. When

this happens, the treadmill should be slowed down or stopped to let the animal relax and regain a more normal gait.

### **C. Factors influencing performance**

**Exercise intensity.** As the animal becomes accustomed to walking on the treadmill, the speed and grade can be increased. Daily sessions of 5–15 minutes are usually sufficient to ensure adequate performance during the experimental session for good runners. For most animals, the speed and grade can be increased to the maximum levels needed for the study after 3–5 days of training. Near-maximal heart rate and cardiac output for a 20- to 25-kg dog can be obtained at 8–10 km/h and 15–20% grade. Dogs in this weight range can trot on the treadmill up to speeds of 8–10 km/h; at higher speeds, the animals gallop and a longer treadmill may be necessary. The dog's workload can be adjusted to obtain the desired percentage of maximal oxygen consumption (279).

### **D. Effects on physiological status**

Dogs undergo the usual adaptations to repeated physical exertion (279, 280), and established protocols for training dogs are available (39, 279, 280). The use of telemetry permits study of “free-ranging” animals, which can help promote a more normal gait as well as more “natural” motivation for severe exertion (377, 380, 381). Obviously, great care should be taken to ensure the safety of the animal and of laboratory personnel in these situations.

# 5 EXERCISE PROTOCOLS USING OTHER SPECIES

## I. OVERVIEW

This chapter reviews general considerations for rabbits, hamsters, guinea pigs, cats, goats, sheep, nonhuman primates, birds, and fish—species that are not widely utilized in studies of the physiology or health impact of exercise. Each section contains brief summaries of studies that use the species in question. General issues regarding the care and use of these animals are addressed in Chapter 2. Readers should review the recommendations for specific exercise modalities (e.g., treadmills, swimming) in other chapters as necessary.

## II. WORKING WITH ATYPICAL SPECIES

### A. Rabbits

Rabbits have been used to study the impact of exercise on various aspects of cardiovascular function and disease. Schedules for training and conditioning rabbits using treadmills have been published (50, 183, 232, 292, 394, 395). A motor-driven wheel has also been used to study the effects of exercise on pacing-induced congestive heart failure. Rabbits were acclimated and trained to exercise on the wheel before undergoing surgery for instrumentation (233). In another study, rabbits were trained to jump over obstacles to reach food and water (83); this approach caused a large number of injuries that would not likely be acceptable to most IACUCs. Swimming has been used to exercise rabbits with sciatic nerve injury (338). However, details concerning implementation of this model were not provided, and the anesthetic regimen used for surgery in this study would not be acceptable to many IACUCs.

Limb immobilization via casting or splints has been used to induce chronic stretch, cartilage degeneration, and osteoarthritis in rabbit hindlimb joints (183, 238). Animal welfare concerns in this model include ensuring proper cast placement to allow adequate mobility and prevent the development

of traumatic injury. Consideration must also be given to growth of young rabbits with casted or splinted limbs, as the size of young rabbits can increase rapidly.

## **B. Hamsters**

The circadian timing of volitional wheel running and the sensitivity to photoperiod and to photic and nonphotic cues have made hamsters an important species for studying the biology and physiology of circadian regulation. Behavioral activation induced by wheel access in hamsters can shift the circadian day phase, entrain rhythms during constant darkness, inhibit hibernation, and modify testosterone levels (256, 303, 315, 389). Access to a running wheel and the associated volitional exercise protects against the spontaneous articular cartilage degeneration that develops in sedentary hamsters (295). Hamsters with access to a running wheel are heavier than sedentary animals because they have greater muscle mass (36).

Hamsters treated with intratracheal elastase are one of the most widely accepted models of human panacinar emphysema (144, 349, 350). Hamsters with emphysema can be trained to walk or run on a treadmill for assessment of exercise during emphysema. Although their spontaneous activity may be equivalent (247), the upper limit of exercise capacity in emphysematous hamsters is much lower than in control animals (94, 342),

Physiological responses to acute exercise have been studied in hamsters during their initial exposure to a treadmill (239). However, animals that are unfamiliar with the treadmill environment and have not developed the necessary motor coordination may experience nonspecific stress that could confound interpretation of the effects of the exercise regimen. Hence, as with other species, hamsters should be familiarized with the treadmill apparatus before actual experimental training and use begins.

## **C. Guinea pigs**

Guinea pigs can be trained to run on a treadmill, although only a small proportion of animals may be sufficiently cooperative (162). Surgical instrumentation should generally be performed only after satisfactory acclimation to the treadmill has been documented for individual guinea pigs (283). Schedules for conditioning and endurance training of guinea pigs are available (162, 284, 376). Guinea pigs may tolerate some forms of exercise

more poorly than other species. For example, swimming for 15 minutes in water maintained at  $25 \pm 2^\circ\text{C}$  caused exhaustion in guinea pigs (71).

Guinea pigs have some novel features that may contribute to their use in exercise studies. Because guinea pigs are precocious neonates, exercise training can begin essentially at birth in this species (326). Like humans, guinea pigs require dietary vitamin C, so they can be used in studies that require dietary control of ascorbate availability. Guinea pigs that are trained to run on a rodent treadmill show exercise-related changes in plasma lipids that are similar to those of humans (97). Because guinea pigs develop atherosclerosis, they may be useful for investigating the beneficial effects of exercise for coronary artery disease (97). Guinea pigs can also be used to study exercise-induced asthma (283, 284).

#### **D. Cats**

Some cats perform volitional aerobic exercise when encouraged by praise and food rewards or by electrical stimulation. However, the behavioral responses of cats forced to exercise on a treadmill with or without electrical stimulation are unpredictable and usually unsuccessful. Nonetheless, even cats with induced spinal injury can be exercised on a treadmill with appropriate attention by the research team (62, 237).

Cats can be used to study the development of muscle hypertrophy through progressive resistance exercise. In such studies, forelimb muscle hypertrophy is induced by requiring cats to pull a weighted lever to obtain food (123). Cats also perform isometric exercise when trained and rewarded with food (203). Finally, the use of measurement devices such as tendon force transducers, intramuscular EMG, and length transducers have been widely used in cats, as this is the most widely used model for determining kinematic and kinetic patterns during locomotion (4, 106, 153, 328).

#### **E. Goats**

Goats will typically perform volitional aerobic exercise for food rewards. Initiating training at a young age, feeding animals while they are on the treadmill, and placing a large mirror in front of them as they exercise can facilitate training. Goats can be motivated to run on treadmills by hand clapping or by touching their hindquarters with a brush. A harness around the thorax that will activate an off switch and suspend the animal if it stumbles

should be used to reduce the potential for injury. Ropes or leashes that are used to lead the goats on and off the treadmill or to maintain their position while they run must not impair circulation or ventilation or cause muscle damage.

Goats that undergo surgery can generally begin treadmill training about 2–4 weeks after surgical instrumentation and before the start of data collection (48, 290). An alternative is to provide pretraining followed by surgery and postrecovery reconditioning.

Physiological responses associated with exercise can vary by breed and age (187). Young (nonruminating) goats on high-fat diets develop fatty lesions in the aorta and can be used to study the effects of diet and exercise on lesion development (317). Goats regulate body temperature by both panting and sweating, which are influenced by hydration status (80, 177, 319).

## F. Sheep

Sheep are commonly used to study maternal-fetal physiology because pregnant ewes develop cardiovascular changes that are similar to those of humans and because the fetus is comparable in size between the two species (325). In sheep and other species, fetal temperature is approximately 0.5°C higher than maternal temperature (214). In sheep, fetal temperature is protected relative to maternal temperature during exercise-induced maternal hyperthermia and during changes in ambient temperature, but this protection is lost during lipopolysaccharide-induced fever (213, 214).

Sheep rely on panting for thermoregulation. Thus thermoregulatory demands contribute to hyperventilation during exercise in sheep. Sheep appear to alter the set point or gain for  $P_{a_{CO_2}}$  regulation during hyperthermic exercise (89, 90). Exposure to ambient temperature of 40°C during exercise raised core temperatures above 41°C in some ewes (213).

In pregnant ewes, hemoconcentration and increased oxygen extraction during exercise maintain uterine  $VO_2$  despite reduced blood flow (235, 236). These responses are likely to maintain fetal  $VO_2$ , and they imply that exercise does not cause a major hypoxic challenge to the fetus (235, 236). In habituated pregnant ewes,  $VO_{2max}$  and lactate levels during exercise can be calculated based on treadmill incline and speed during 5-minute exercise bouts (181).



Elevations in heart rate also provide an index of exercise intensity in sheep (311). In one study sheep were trained and conditioned over a period of weeks to exercise at 2.1 km/h and inclines of 5° or 10° for 30 minutes (213), but other investigators submitted ewes to a similar regimen without conditioning in studies measuring maternal and fetal hormones and regional blood flow during exercise (26, 27).

## **G. Nonhuman primates**

Not all primates will perform volitional exercise, but they usually can be trained to move their arms or legs while confined to a chair, use a bipedal treadmill while grasping a support bar with the forelimbs, run on all fours inside an enclosed ventilated treadmill, or run in a large motor-driven, enclosed revolving drum (173, 312, 390). Animals must be adapted to the testing environment and closely monitored while they exercise because they are prone to injury if they are unable or unwilling to perform the task. Using telemetry rather than tethers or other external devices to collect physiological data (145, 314, 354) can reduce problems associated with external data collection devices. Food rewards can greatly increase compliance during seated exercise protocols.

As in other species, environmental temperature is an important variant in studies of exercise in nonhuman primates. In particular, rhesus monkeys have a lower maximal rate of sweating than humans (119, 120, 178). This lower limit can compromise tolerance to exercise in hot environments. For example, trained rhesus monkeys were able to successfully complete six work/rest cycles (10-min work, 1-min rest at a minimum of 3 mph) at environmental temperatures of 15°C and 25°C but could only complete three cycles at 35°C because of excessive heat retention that resulted in core body temperature in excess of 40°C (145). In contrast, the sweating rate of patas monkeys more closely resembles that of humans (120), whereas squirrel monkeys rely on both behavioral responses and limited sweating to promote heat loss (358, 359).

USDA requirements to provide environmental enrichment for nonhuman primates have given these animals a variety of exercise opportunities but concurrently reduce environmental uniformity across and even within institutions. Volitional exercise can variably alter many physiological and behavioral variables among individual animals. For instance, as in humans, the extent to which nonhuman primates maintain

lower heart rates at rest and after exercise varies as a function of physical conditioning (208). Because of this potential for influencing physiological responses, enrichment-related opportunities for volitional exercise should always be described accurately in reports on studies involving these animals, particularly if exercise is an experimental parameter.

Caged nonhuman primates may pace, walk in circles, or perform other repetitive stereotyped movements that may be vigorous or prolonged enough to cause physiological effects that are not representative of an entire study population. Such animals may be inappropriate for use in exercise studies.

## **H. Birds**

The exercise capabilities of birds can be studied while they fly in wind tunnels, run on treadmills, swim, or dive (339). Some species, like chickens, run readily on treadmills, whereas others are unwilling to run at high speeds and repeatedly attempt to fly (probably reflecting the inherent nature of the species in a natural setting) (122). Treadmills must be enclosed to prevent injuries in birds that attempt to fly. To improve traction and reduce sliding, the treadmill belt should be covered with nonskid tape. Avian toenails are prone to injury, bleeding, and potential infection with treadmill running. Clipping toenails before exercise, applying disinfectant to any toenails that become injured to prevent infection, and disinfecting mats can reduce these problems.

Chickens running on treadmills respond more readily to touching of the tail feathers than to electrical stimulation as a motivator. To stimulate performance, investigators can also suspend balls of tape near the end of the treadmill, place mirrors in front of the chickens, and position transparent Plexiglas walls between lanes. Training for 2–3 weeks will produce a consistent steady-state ventilatory response in domestic fowl during exercise (122).

Muscular hypertrophy can be induced in chickens and quail by attaching a weight to one wing (238). The use of fully grown animals for muscle hypertrophy studies simplifies data analysis because muscle hypertrophy is not confounded by normal muscle growth (238). Continual stretch is not necessary to induce hypertrophy. In one study, for example, stretch applied for only 30 minutes per day induced about 50% of the increase in muscle mass that accrued in response to 8 hours of stretch per day (24).

Numerous characteristics of avian respiratory anatomy and physiology differ substantially from mammalian systems. For instance, birds have indistensible lungs, unidirectional ventilation for gas exchange, and intrapulmonary CO<sub>2</sub> receptors (122). When they fly, birds increase oxygen delivery by increasing their heart rate, with little change in oxygen extraction or cardiac stroke volume (43). The physiological characteristics and specializations of birds also vary widely across species. Birds use hyperventilation to control both temperature and oxygen delivery. Temperature can also influence acid-base balance during exercise-induced hyperventilation in birds. Therefore, as in mammalian studies, ambient temperature must be carefully controlled in studies involving birds.

## I. Fish

The growing availability of genetically modified zebra fish increases the likelihood that fish will be used increasingly in exercise physiology and functional genomics (305). As a result, IACUCs and PIs will probably need to consider exercise in fish more frequently in the future.

Body size, temperature, diet and nutritional status (food quantity and quality), water quality, handling, and training can all influence the ability of fish to perform and recover from exhaustive exercise. Constraints placed on a fish before and during exercise (e.g., food deprivation) can cause large intraspecies variation in physiological responses (190). Although periods of food deprivation are common in the lives of many fish in natural settings, this practice can influence physiological and biochemical processes that could affect metabolism during swimming (191, 382).

Fish have a single circulatory system, with a single atrium and ventricle that pump blood through the gills for gas exchange. During exercise (forced swimming in water channels), trout and cod increase oxygen consumption through increased oxygen extraction and cardiac stroke volume, with little change in heart rate (43).

Selection of the ideal control situation for comparison to exercised animals is complex. Control (nonexercised) fish are commonly kept in a blackened box, but limiting movement can itself alter metabolite levels in fish (190, 260). The rate of recovery from exhaustive exercise depends, to a large extent, on which metabolite is being measured (273).



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# APPENDIX A

## Hindlimb Suspension and Immobilization of Rats and Mice

### *Hindlimb suspension model*

The hindlimb suspension model for eliminating the locomotive function of the hindlimbs of rats and mice was initially developed in the 1970s at the Ames Research Center of the National Aeronautics and Space Administration as a terrestrial model of the microgravity environment during spaceflight (270, 271). It has since been used in numerous studies of simulated weightlessness and other aspects of musculoskeletal unloading.

In this model, the animal has a cast applied to the base of the tail and is placed in a 30–45° head-down tilt, thereby removing the weight-bearing function of the hindlimbs and inducing a cephalic fluid shift. Care should be taken to ensure that the cast is only applied to the base of the tail, as the rat uses the tail in thermoregulation and coverage of the remainder of the tail may impair this important function (272).

Several excellent reviews of the metabolic and neuromuscular adaptations induced by this model have been published (3, 367). In addition, the interested reader can refer to the comprehensive review of the technical aspects of the hindlimb suspension model by Morey-Holton and Globus (272). This appendix is intended to serve as a reference for researchers, reviewers of manuscripts, and IACUCs, and it covers several important aspects of the model. It also includes a history of the hindlimb suspension technique in rats and important technical details of the use of the model, including considerations of housing, room temperature, unloading angle, the potential need for multiple control groups, age, body weight, the use of the forelimb tissues as internal controls, criteria for the removal of animals from suspension experiments, and how physiological responses caused by the model compare with the adaptive responses induced by actual spaceflight in rats. Moreover, this section discusses the extension of the hindlimb suspension model to mice.

An important aspect of the hindlimb suspension model not covered in the review by Morey-Holton and Globus (272) is the use of wire-bottomed cages. Wire-bottomed cages improve restriction in mobility and permit more accurate in-time monitoring of stress by allowing a more rapid and accurate

determination of food intake and fecal production. Wire-bottomed cages themselves do not seem to alter experimental any more than randomized differences among pine shavings, eucalyptus pulp, vermiculite, and wire-bottomed cages (310). Despite differences in weight, cage type, and supplier, foot lesions were not found in rats until the animals had been housed for more than 1 year (300). Rats individually housed in wire-bottomed caging before urine collection can consume and excrete a larger quantity of water than rats group housed in solid-bottomed cages (227).

### ***Hindlimb immobilization model***

Hindlimb immobilization in rats and mice mimics the immobilization of human limbs, a common clinical procedure performed in hospitals and clinics. This model elicits the absolute removal of electrical and mechanical activity of the locomotor muscles of the hindlimb and is used primarily in investigations of the mechanisms of skeletal muscle atrophy in the immobilized limbs (35, 100). As there is no single resource describing the technical aspects of limb immobilization in rodents, a discussion of this topic is provided below.

- ***Anesthesia.*** Rats and mice must be anesthetized so that no foot pinch reflex occurs. Older rats will have better recovery from anesthesia when isoflurane is used. When a cocktail of ketamine-xylazine-acepromazine is used, a small percentage of aged rats may never fully recover from anesthesia and are prone to hypothermia. With ketamine anesthesia alone, rats tend to have withdrawal reflexes during application of the plaster cast.
- ***Casting procedures.*** Plaster of Paris can be used to form the cast. Plaster of Paris sets faster when warm water is used, and a dedicated container should be used to wet the plaster because the container will gain plaster during the wetting and squeezing process and cannot be used for anything else. One option is to use the plaster of Paris sold by medical supply companies for human patients (e.g., Johnson & Johnson Specialist Plaster Bandages: Fast Setting). The investigator cuts the product into 5- and 2.5-cm-wide strips of varying lengths (e.g., 15 and 45 cm). Faster-drying casting material that is not plaster of Paris may be problematic, because it sets before it can be completely applied to the hindlimbs, a process that takes about 15 minutes. To speed the drying process, water should be squeezed out of the plaster of Paris before application.

For casting of the limb, the rat or mouse should be placed with its ventral side up and its head tilted away from the side of the casted leg. This maneuver adjusts the skin near the coxo-femoral joint (hereafter designated as the hip) into a more optimal position for application of the plaster of Paris. Be certain the airway is not obstructed.

1. The first plaster strip (~2.5 cm wide) is applied over the thigh, pulling firmly over hip joint so there is no slack in the strip but ensuring that it is not tight. Place material closer to the hip than to the stifle or femoral-tibio-patellar joint (hereafter designated as the knee).
2. The second plaster strip (~2.5 cm wide) is applied from the lateral thigh along the back of the calf muscles, continuing to wrap around the foot, and then wrapped back toward the medial side of the thigh. If the tibio-tarso-metatarsal joint or hock (hereafter designated as the ankle) is fixed in *plantar flexion*, the knee should be fixed at about a 90° angle. If the ankle is fixed in *dorsiflexion*, the knee should be fixed at close to an 180° angle.
3. A shorter third plaster strip (~2.5 cm wide) is applied to the back of the leg to connect/reinforce the overlapping second strip on its lateral and medial sides on the back of the leg.
4. An initial approximation of the angle at which the ankle is fixed is made at this point. Fixation of the muscle in a shortened position will result in atrophy, whereas fixation in a lengthened position either prevents or attenuates the atrophy. If the ankle is fixed in *plantar flexion*, the calf muscles will be fixed in a shortened position and the tibialis anterior muscle will be fixed in a stretched position. Fixation of the ankle to produce *dorsiflexion* will reverse the lengths of the muscles from plantar flexion. Dorsiflexion produces stretching of the calf muscles and shortening of the tibialis anterior muscle.

If the goal is quadriceps atrophy, the animal should be casted with slight hip flexion and knee extension. Knee extension will also lengthen the gastrocnemius muscle.

It is critical that the plaster strip not be applied over the dorsal aspect of the ankle. In addition, the plaster of Paris should not be applied to the proximal third of the dorsal aspect of the foot, over

the dorsal aspect of the ankle, and the distal third of the cranial aspect of the crus (over the tibialis anterior muscle), as this could obstruct blood flow to the foot.

It is preferable to cover the toes with plaster, because open toes can be injured when the animal uses its front legs to move around the cage. However, if the investigator is attempting this procedure for the first time, it is advisable to leave the toes uncovered in order to monitor toe coloration. Because the primary goal is to fix the ankle in the final desired position, an additional one or two strips may be applied so that the cast on the leg forms a single piece.

5. The cast on one leg should be allowed to dry before the cast is applied to the opposite leg. If the contralateral leg is casted before the ipsilateral leg's cast is set, the position of the ankle of the ipsilateral leg may change. Warm air from a hair dryer can be used to speed drying (however, hot air could burn the skin). Squeezing water out of the plaster of Paris will accelerate the drying process. When the first leg is fixed so that moving the rat does not change the ankle's angle, the investigator may proceed to the opposite leg and repeat the above procedure.
6. When the plaster on the second leg dries to the degree that its ankle and knee joints are fixed, the animal can be gently rotated so that it is dorsal side up. The ankle should again be checked to ensure that it remains fixed in the appropriate position.
7. The thighs should be connected with a plaster strip (~5 cm wide) across the back without causing straightening of the spine. The hip angle should be positioned such that genital area is about 1 cm above the wire-bottomed cage (i.e., the cast material over the knee should be in contact with the bottom of the cage, whereas the lower abdomen should be slightly elevated).
8. The next step is to form a wire mesh "sandwich," using the existing layer of plaster as the bottom layer over which ¼-inch galvanized wire mesh is placed in strips (2.5 cm wide over the plaster covering the back and 0.5 cm wide over the plaster covering the calf muscles). The investigator should be certain that there are no sharp projections on the wire mesh that might harm the animal or cause irritation.
9. Next, a long strip (~5 cm wide) of plaster is layered over the wire mesh strips to reinforce the existing cast. The plaster strip should cover the back, wrapping first over the back and then around both

thighs, to further secure the thigh by tightening around the upper portion of the thigh. The loose skin covering the upper thigh usually prevents a tight fit when the first plaster strip encircles the thigh. If the previous plaster casting material at the proximal thigh is not fitting tightly, the investigator can gently break the solid cast (making it flexible), so that the next strips over the back and around the thighs fit more tightly.

- *Recovery from anesthesia.* Young and mature rats should be monitored closely in their cages during the recovery period. Older rats (e.g., 24–30 mo old) will likely require a heating pad to assist maintenance of body temperature. If isoflurane anesthetic is used, rats normally wake up within minutes.
- *Food and water.* It is important to ensure that food is always placed at the bottom of the cage (improving food access) and that the immobilized animal is able to reach the water dispenser without the dispenser interfering with the animal's movement.
- *Additional considerations.* The following occurrences may be problematic and should be taken into consideration when using the limb immobilization procedure. Potential solutions to these problems are suggested:
  1. One concern is that plastic-bottomed cages may not allow the immobilized rat to move around easily and may induce food deprivation, dehydration, and distress, even when bedding materials are placed in the bottom of the cage. One suggested solution is the use of wire-bottomed caging for animals undergoing hindlimb immobilization. This will allow the casted animal to use its forelimbs to pull itself around the cage without any observable problems, and a stress response is not induced, as no adrenal hypertrophy or gastric ulcers have been observed (35).
  2. Another potential problem that can arise with casted rodents is that the animal may chew the cast. The investigator should check the cast daily for evidence of gnawing or chewing. Chewing will ensue even if noxious materials are applied to the cast to prevent this activity. If chewing of the cast occurs, the chewed spot should be patched with additional sheets of plaster. The most frequent area for chewing is around the feet. Patching can be done by having the rat walk into a thick glove and holding the rat in the glove while patching the cast. If done properly, this alleviates having to

anesthetize the rat, which would be a greater physiological stress than a short time inside a thick glove dedicated to this process.

3. If the animal appears frustrated, the investigator may add wooden dowels as an enrichment, to give the animal an alternative chewing source.
4. In male rats, there may be irritation of the genital area due to the penis rubbing on the floor of a wire-bottomed cage. One solution is to produce the appropriate hip angle so that the genital area is about 1 cm above the wire-bottomed cage (i.e., the cast material over the knee should be in contact with the bottom of the cage, whereas the lower abdomen should be slightly elevated).
5. If the skin rubs on the wire mesh, the investigator should trim the wire mesh of all projections, so that if the rat chews into the wire mesh, a projection from a cut section of mesh does not poke into the skin.
6. Food intake can be impacted by the immobilization process. Immobilized rats will eat less the first day after casting and will then progressively increase their daily food intake. In some cases, older rats may become depressed and stop eating. The investigator should monitor food and water intake of all animals. The belly of the animal can also be palpated. Food intake can be improved by placing apple juice on the food and on wooden dowels cut into 10-cm segments. Group housing of rats may help with alleviating depression and enhancing food consumption.
7. Ulceration from cast rubbing may occur as early as the 14th day of hindlimb immobilization because of shrinkage of limb diameter due to skeletal muscle atrophy and the smaller limb moving within the cast. The cast should be replaced every 14 days. If sores develop, the limb should be wrapped in a bandage before placement of the new cast.
8. In some cases, blood flow to the foot may be limited. If plaster of Paris is placed over the distal aspect of the ankle blood flow could be cut off to the foot, and if this persists, the foot will darken. Care should be taken not to apply plaster of Paris to the top of the ankle.
9. If the spine is fixed during the casting procedure and not allowed to retain its normal curvature, discomfort could potentially be induced. To avoid this problem, the investigator should ensure that the back straps of plaster of Paris are not too tight. One should be able to

view the normal convex curvature of the spine from the dorsal aspect of the animal.

10. On rare occasions, the casting material can compress into the pelvic region and cause penile protrusion. If this happens, compression of the casting material pressing into the pelvic region should be relieved.

When attention and appropriate action are given to the foregoing potential problems, stress to the casted animal is minimized and no adrenal hypertrophy or gastric ulcers are noted (35).





# APPENDIX B

## Sample Animal Exercise Protocol Scenarios for Institutional Animal Care and Use Committees (IACUCs) and Principal Investigators

Described below are three hypothetical animal exercise protocols submitted by a principal investigator (PI) to an institutional animal care and use committee (IACUC). Some relevant “considerations” related to various aspects of the proposed protocols that should be taken into account by both the PI and IACUC members are also listed. Many of the points raised in these scenarios would also be relevant to a PI when designing animal exercise procedures, as well as to journal editors and reviewers when evaluating manuscripts that contain these types of protocols.

### SCENARIO #1

1. Young (2-mo-old) male Sprague-Dawley rats will be used to evaluate gene expression profiles in forelimb and hindlimb skeletal muscles in response to aerobic exercise training.
2. A total of 20 rats will be used. Rats will be randomly assigned to either a control ( $n = 10$ ) or an exercise ( $n = 10$ ) group.

#### *Considerations:*

- Were power calculations performed to determine an adequate “n” for each group?
  - The PI must appreciate that all rats in the exercise group may not be suitable runners (as many as 10% of rats purchased from commercial vendors refuse to walk/run)—additional rats should be added to this group to account for drop-out so that an adequate number of animals is available at the end of the training period
3. Because rats enjoy running, they will not need to be familiarized with a motorized rodent treadmill before the start of the exercise training protocol. The protocol will initially consist of running rats continuously for a 45-minute period at moderate speeds. Treadmill speeds will gradually build up to 90 minutes per session.

***Considerations:***

- Familiarization with running on the treadmill must be part of the protocol.
  - A typical time frame for familiarization is 5–14 days, consisting of 5–15 minutes per session at varying treadmill speeds.
  - A duration of running of 45 minutes is too long as a starting point for a training protocol.
  - “Moderate speeds” is a vague term and needs to be more specifically defined.
  - If using a motorized treadmill, the investigator may employ an aversive stimulus (electric grid) to keep animals exercising. The electric grid should be:
    - Noxious enough (i.e., high enough voltage) to provide sufficient incentive to keep the animal running
    - Mild enough (low amperage) not to harm the animal
    - An alternative to a shock grid: high-pressure air directed toward the animal
4. Rats will run 7 days per week for a period of 6 months to ensure that an aerobic “training effect” is achieved. When animals show signs of fatigue during an exercise bout, the aversive stimulus (electric shock grid) will be increased. Rats will be inspected for injuries once every 2 weeks during the training period.

***Considerations:***

- What is the justification for exercising rats 7 days per week?
- What is the justification for a training duration of 6 months? Could the training effects of interest be manifested over a shorter time frame?
- What is the investigator’s marker(s) for determining whether there was an aerobic training adaptation?
- Guidelines for dealing with aversive stimuli should be clearly described.
- Rats should be inspected daily when they are involved in a training study.
- At the end of the training period, it is typical to measure “endurance capacity.” In this scenario, time to “fatigue” would typically be used

as a marker of endurance capacity, and fatigue would be defined as an inability to keep pace with the treadmill. Animals should be removed at the point when fatigue is reached.

5. Because rats quickly adapt to treadmill running and can run continuously for prolonged periods if “appropriate” levels of an aversive stimulus are available, supervision will consist of a technician checking on animals once every 10–15 min during a training session.

***Considerations:***

- Knowledgeable personnel should continually monitor animals during exercise sessions, particularly in the early phases of a conditioning program and near the end of training sessions.
  - Behavioral or physiological markers may be identified that can alert the observer that the trial must be terminated or the demands reduced.
6. The treadmill training sessions will be conducted in a small room adjacent to the PI’s main laboratory.

***Considerations:***

- Does this space have adequate ventilation and temperature control? Increased ambient temperature can place significant thermoregulatory and cardiovascular strain on rats.

## **SCENARIO #2**

1. A PI wants to compare the gene expression profile in skeletal muscles of rats in response to different modes of exercise, hypothesizing that different muscle recruitment patterns would alter gene expression responses. Therefore, the PI proposes a swim training study in rats to complement motorized treadmill experiments, as these two modes of exercise will elicit different muscle recruitment patterns.
2. The PI proposes to use a shallow rectangular tank (depth and dimensions not specified) filled to the top with water (temperature not specified).

***Considerations:***

- Swimming behaviors likely to be observed in rats include floating (trapping of air bubbles in fur can increase buoyancy and is a learned

behavior), climbing, diving, and bobbing. These behaviors can induce hypoxia, and the data may not reflect the effects of swimming per se.

- An observer should be present at all times to prevent drownings.
- Criterion to stop a session: submersion time (e.g., >3 seconds if a rat is not bobbing or diving).
- Depth of the tank should be >50 cm to prevent bobbing.
- Tank design: edges of the tank should be round (or rats will be able to hang in corners).
- Water height: distance from waterline to top of tank should be sufficient to prevent rats from pulling themselves up and out of the water.
- Water temperature: should be between 33 and 36°C. Animals should be towel-dried before returning to cooler ambient temperatures.
- For treadmill training studies, it is possible to increase speed, duration, or grade as a means to increase workload and continue to provide a training stimulus. With swimming, this is difficult, although it is possible to attach small weights to a rat's tail to increase the workload.

### SCENARIO #3

The PI wants to study knockout mice that are deficient in a mitochondrial enzyme involved in aerobic metabolism to gain additional insight into mechanisms regulating skeletal muscle gene expression during exercise.

**Question:** With regard to exercise protocols, is it appropriate to consider a mouse just a small version of a rat?

**Answer:** No

#### *Considerations:*

- Strain differences exist among mice for performance in treadmill running, wheel running, and swimming (e.g., C57BL/6J mice are poor runners).
- A rat treadmill can be used, but the lanes are relatively wide and mice will expend substantial energy on lateral movement. Therefore, a narrower lane is recommended.
- During swimming, mice spend less time bobbing, diving, or climbing compared to rats. Also, mice primarily use hindlimbs in swimming (rats use both hindlimbs and forelimbs).

- The ratio of surface area to mass is much greater in a mouse than a rat, which is important for thermoregulation in both running and swimming paradigms.



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