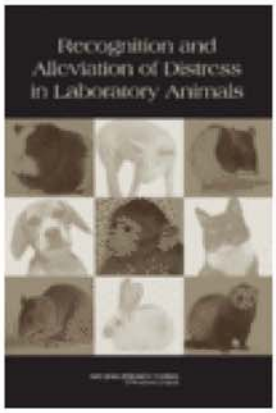


Recognition and Alleviation of Distress in Laboratory Animals

Committee on Recognition and Alleviation of Distress in Laboratory Animals, National Research Council

ISBN: 0-309-10818-7, 132 pages, 6 x 9, (2008)

Recognition and Alleviation of Distress in Laboratory Animals

Committee on Recognition and Alleviation of Distress in Laboratory Animals

Institute for Laboratory Animal Research

Division on Earth and Life Studies

NATIONAL RESEARCH COUNCIL
OF THE NATIONAL ACADEMIES

THE NATIONAL ACADEMIES PRESS
Washington, D.C.
www.nap.edu

THE NATIONAL ACADEMIES PRESS 500 Fifth Street, N.W. Washington, DC 20001

NOTICE: The project that is the subject of this report was approved by the Governing Board of the National Research Council, whose members are drawn from the councils of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine. The members of the Committee responsible for the report were chosen for their special competences and with regard for appropriate balance.

This study was supported by the American Veterinary Medical Association; Aventis Pharmaceuticals; the Bosack-Kruger Foundation; Bristol-Myers Squibb; GlaxoSmithKline; Humane Society of the United States; Scientists Center for Animal Welfare; Wyeth-Ayerst Pharmaceuticals; Department of Agriculture, Animal and Plant Health Inspection Service; and Department of Health and Human Services, National Institutes of Health through Contract Number N01-OD-4-2139 Task Order #161. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the organizations or agencies that provided support for the project. The content of this publication does not necessarily reflect the views or policies of the National Institutes of Health, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Library of Congress Cataloging-in-Publication Data

Recognition and alleviation of distress in laboratory animals /
Committee on Recognition and Alleviation of Distress in Laboratory Animals, Institute for Laboratory Animal Research, Division on Earth and Life Studies.
p. ; cm.
Includes bibliographical references and index.
ISBN-13: 978-0-309-10817-1 (pbk.)
ISBN-10: 0-309-10817-9 (pbk.)
1. Laboratory animals—Health. 2. Laboratory animals—Effect of stress on. 3. Animal welfare. I. Institute for Laboratory Animal Research (U.S.). Committee for Recognition and Alleviation of Distress in Laboratory Animals.
[DNLM: 1. Animal Welfare—standards. 2. Animals, Laboratory. 3. Laboratory Animal Science—ethics. QY 54 R307 2008]
SF406R43 2008
636.088'5—dc22
2007052927

Additional copies of this report are available from
The National Academies Press
500 Fifth Street, NW
Box 285
Washington, DC 20055

800-624-6242
202-334-3313 (in the Washington metropolitan area)
http://www.nap.edu

Printed in the United States of America

THE NATIONAL ACADEMIES

Advisers to the Nation on Science, Engineering, and Medicine

The **National Academy of Sciences** is a private, nonprofit, self-perpetuating society of distinguished scholars engaged in scientific and engineering research, dedicated to the furtherance of science and technology and to their use for the general welfare. Upon the authority of the charter granted to it by the Congress in 1863, the Academy has a mandate that requires it to advise the federal government on scientific and technical matters. Dr. Ralph J. Cicerone is president of the National Academy of Sciences.

The **National Academy of Engineering** was established in 1964, under the charter of the National Academy of Sciences, as a parallel organization of outstanding engineers. It is autonomous in its administration and in the selection of its members, sharing with the National Academy of Sciences the responsibility for advising the federal government. The National Academy of Engineering also sponsors engineering programs aimed at meeting national needs, encourages education and research, and recognizes the superior achievements of engineers. Dr. Charles M. Vest is president of the National Academy of Engineering.

The **Institute of Medicine** was established in 1970 by the National Academy of Sciences to secure the services of eminent members of appropriate professions in the examination of policy matters pertaining to the health of the public. The Institute acts under the responsibility given to the National Academy of Sciences by its congressional charter to be an adviser to the federal government and, upon its own initiative, to identify issues of medical care, research, and education. Dr. Harvey V. Fineberg is president of the Institute of Medicine.

The **National Research Council** was organized by the National Academy of Sciences in 1916 to associate the broad community of science and technology with the Academy's purposes of furthering knowledge and advising the federal government. Functioning in accordance with general policies determined by the Academy, the Council has become the principal operating agency of both the National Academy of Sciences and the National Academy of Engineering in providing services to the government, the public, and the scientific and engineering communities. The Council is administered jointly by both Academies and the Institute of Medicine. Dr. Ralph J. Cicerone and Dr. Charles M. Vest are chair and vice chair, respectively, of the National Research Council.

www.national-academies.org

COMMITTEE ON RECOGNITION AND ALLEVIATION OF DISTRESS IN LABORATORY ANIMALS

Members

Peter A. Ward *(Chair)*, University of Michigan Medical School
Robert Blanchard, University of Hawaii
Valerie Bolivar, New York State Department of Health
Marilyn J. Brown, Charles River Laboratories
Fon Chang, AstraZeneca, Boston
James P. Herman, University of Cincinnati
Robert Hubrecht, Universities Federation for Animal Welfare
David M. Lawson, Wayne State University School of Medicine (retired)
Steven F. Maier, University of Colorado
David Morton, University of Birmingham, United Kingdom (retired)
Steven M. Niemi, Massachusetts General Hospital
Melinda A. Novak, University of Massachusetts
Stephen L. Zawistowski, American Society for the Prevention of Cruelty to Animals

Staff

Joanne Zurlo, Director
Lida Anestidou, Study Director (from November 2006)
Marsha Barrett, Former Project Assistant (to September 2006)
Kathleen Beil, Administrative Coordinator
Cameron Fletcher, Senior Editor (from June 2007)
Rhonda Haycraft, Senior Project Assistant (from September 2006)
Jennifer Obernier, Former Study Director (to August 2006)
Susan Vaupel, Former Senior Editor (to June 2007)
Shimere Williams, Mirzayan Fellow

INSTITUTE FOR LABORATORY ANIMAL RESEARCH COUNCIL

Members

Stephen W. Barthold *(Chair),* University of California, Center for Comparative Medicine, Davis, California
Kathryn A. Bayne, Association for Assessment and Accreditation of Laboratory Animal Care International, Waikoloa, Hawaii
Myrtle A. Davis, Lilly Research Laboratories, Greenfield, Indiana
Jeffrey I. Everitt, GlaxoSmithKline Research and Development, Comparative Medicine and Investigator Support, Research Triangle Park, North Carolina
Michael F. Festing, Leicestershire, United Kingdom (to June 2006)
James G. Fox, Massachusetts Institute of Technology, Division of Comparative Medicine, Cambridge, Massachusetts
Nelson L. Garnett, Consultant, Laboratory Animal Care and Use Programs, Dickerson, Maryland
Estelle B. Gauda, Johns Hopkins University School of Medicine, Johns Hopkins Hospital, Baltimore, Maryland
Coenraad F.M. Hendriksen, Netherlands Vaccine Institute, Bilthoven, The Netherlands
Jon H. Kaas, Vanderbilt University, Nashville, Tennessee
Joseph W. Kemnitz, University of Wisconsin, Primate Research Center, Madison, Wisconsin
Judy A. MacArthur Clark, (formerly) Pfizer Global R&D, Groton, Connecticut
Leticia V. Medina, Abbott Laboratories, Abbott Park, Illinois
Bernard E. Rollin, Colorado State University, Department of Animal Sciences, Fort Collins, Colorado
Abigail L. Smith, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, Pennsylvania
Stephen A. Smith, Virginia Polytechnic Institute and State University, Department of Biomedical Sciences and Pathobiology, Blacksburg, Virginia
Peter Theran, Massachusetts Society for the Prevention of Cruelty to Animals, Boston, Massachusetts (to January 2007)
James E. Womack, Texas A&M University, College Station, Texas

Staff

Joanne Zurlo, Director
Lida Anestidou, Program Officer
Kathleen Beil, Administrative Coordinator
Cameron Fletcher, Managing Editor, *ILAR Journal*
Rhonda Haycraft, Senior Project Assistant

INSTITUTE FOR LABORATORY ANIMAL RESEARCH PUBLICATIONS

Toxicity Testing in the 21st Century: A Vision and a Strategy (2007)
Overcoming Challenges to Develop Countermeasures Against Aerosolized Bioterrorism Agents: Appropriate Use of Animal Models (2006)
Guidelines for the Humane Transportation of Research Animals (2006)
Science, Medicine, and Animals: Teacher's Guide (2005)
Animal Care and Management at the National Zoo: Final Report (2005)
Science, Medicine, and Animals (2004)
The Development of Science-based Guidelines for Laboratory Animal Care: Proceedings of the November 2003 International Workshop (2004)
Animal Care and Management at the National Zoo: Interim Report (2004)
National Need and Priorities for Veterinarians in Biomedical Research (2004)
Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003)
International Perspectives: The Future of Nonhuman Primate Resources, Proceedings of the Workshop Held April 17-19, 2002 (2003)
Occupational Health and Safety in the Care and Use of Nonhuman Primates (2003)
Definition of Pain and Distress and Reporting Requirements for Laboratory Animals: Proceedings of the Workshop Held June 22, 2000 (2000)
Strategies That Influence Cost Containment in Animal Research Facilities (2000)
Microbial Status and Genetic Evaluation of Mice and Rats: Proceedings of the 1999 US/Japan Conference (2000)
Microbial and Phenotypic Definition of Rats and Mice: Proceedings of the 1998 US/Japan Conference (1999)
Monoclonal Antibody Production (1999)
The Psychological Well-Being of Nonhuman Primates (1998)
Biomedical Models and Resources: Current Needs and Future Opportunities (1998)
Approaches to Cost Recovery for Animal Research: Implications for Science, Animals, Research Competitiveness and Regulatory Compliance (1998)
Chimpanzees in Research: Strategies for Their Ethical Care, Management, and Use (1997)
Occupational Health and Safety in the Care and Use of Research Animals (1997)
Guide for the Care and Use of Laboratory Animals (1996)
Guide for the Care and Use of Laboratory Animals—Korean Edition (1996)

Guide for the Care and Use of Laboratory Animals—Chinese Version (1996)
Guide for the Care and Use of Laboratory Animals—Spanish Version (1996)
Guide for the Care and Use of Laboratory Animals—Russian Version (1996)
Guide for the Care and Use of Laboratory Animals—French Version (1996)
Guide for the Care and Use of Laboratory Animals—Taiwanese Edition (1996)
Guide for the Care and Use of Laboratory Animals—Portuguese Edition (1996)
Guide for the Care and Use of Laboratory Animals—Japanese Edition (1996)
Rodents (1996)
Nutrient Requirements of Laboratory Animals, Fourth Revised Edition (1995)
Laboratory Animal Management: Dogs (1994)
Recognition and Alleviation of Pain and Distress in Laboratory Animals (1992)
Education and Training in the Care and Use of Laboratory Animals: A Guide for Developing Institutional Programs (1991)
Companion Guide to Infectious Diseases of Mice and Rats (1991)
Infectious Diseases of Mice and Rats (1991)
Immunodeficient Rodents: A Guide to Their Immunobiology, Husbandry, and Use (1989)
Use of Laboratory Animals in Biomedical and Behavioral Research (1988)
Animals for Research: A Directory of Sources, Tenth Edition and Supplement (1979)
Amphibians: Guidelines for the Breeding, Care and Management of Laboratory Animals (1974)

Copies of these reports may be ordered from the National Academies Press
(800) 624-6242 or (202) 334-3313
www.nap.edu

Preface

The impetus for this project was a letter from the New Jersey Association for Biomedical Research requesting that the National Academies' Institute for Laboratory Animal Research (ILAR) form a Committee to update its 1992 report *Recognition and Alleviation of Pain and Distress in Laboratory Animals*. More than a decade had passed since publication of the initial report, and many in the laboratory animal community felt that scientific progress in the areas of pain and distress warranted an update, as there was little guidance to assist investigators, laboratory animal veterinarians, animal care staff, and animal care and use committee (IACUC) members in assessing whether a proposed protocol would cause distress or whether an animal was experiencing distress. Current literature dealing with the development and recognition of stress and distress in other vertebrates, such as fish, is similarly very limited. Although there is reasonable consensus regarding the clinical signs of stress and distress, there are mixed views as to whether stress and distress develop independently of each other or whether the latter derives from the former. Much more information is still needed.

The panel of experts that prepared this report has endeavored to present its best understanding of the diagnosis and treatment of stress and distress, based on peer-reviewed published literature. This report represents a consensus of experts who have described areas where there seems to be reasonable agreement as well as areas where there is inadequate knowledge, indicating the need for future research. The Committee was challenged to adopt a consistent terminology and define the subjects of the report. In deference to extensive deliberations and varied interpretations of the available literature, and in the name of achieved consensus, the Committee refrained from

proposing any definitions. Moreover, due to inadequate relevant scientific information, the report references the Committee's best professional judgment and expert opinion in areas where further research is needed. We believe that the outcome reflects a balanced exposition of where this field currently stands. The Committee hopes this report will be useful to all who are involved in the care and use of laboratory animals.

The Committee acknowledges the individuals who provided assistance and valuable information for our deliberations. At the first meeting of the Committee, on April 10, 2006, a group of experts made presentations that addressed policy implications and covered numerous perspectives on the concept of laboratory animal distress. Specifically, the Committee thanks:

Joseph Garner, Purdue University
J.R. Haywood, Michigan State University, East Lansing
Philip V. Holmes, University of Georgia
Michael D. Oberdorfer, National Eye Institute, NIH
Andrew N. Rowan, Humane Society of the United States
Michael Scheeringa, Tulane University

Two additional speakers addressed the Committee at its meeting on September 6, 2006, and the Committee thanks them as well:

Roland Anderson, The Seattle Aquarium
James D. Rose, University of Wyoming

This report has been reviewed in draft form by individuals chosen for their diverse perspective and technical expertise, in accordance with procedures approved by the Report Review Committee of the National Research Council (NRC). The purpose of this independent review is to provide candid and critical comments that will assist the Committee in making its published report as sound as possible and to ensure that the report meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the deliberation process. The Committee thanks the following individuals for their review of this report:

Donald M. Broom, University of Cambridge
Joy Cavagnaro, Access BIO
Mary Dallman, University of California San Francisco
Michael Festing, University of Leicester (*retired*)
Monika Fleshner, University of Colorado Boulder
Joseph P. Garner, Purdue University
Barbara Hansen, University of South Florida

Randall J. Nelson, University of Tennessee Health Science Center
Glen Otto, University of Texas at Austin
Cynthia Pekow, VA Puget Sound Health Care System
Jeremy Turner, Illinois College and Southern Illinois University

The review of the report was overseen by:

Hilton J. Klein, Merck Research Laboratories (*retired)*
Harley W. Moon, Iowa State University (*emeritus*)

Appointed by the NRC, these individuals were responsible for making certain that an independent examination of this report was carried out in accordance with institutional procedures and that all review comments were carefully considered. Responsibility for the final content of this report rests entirely with the authoring Committee and the institution.

I also extend my deep appreciation to the Committee members and staff who devoted considerable time to this report. In particular I would like to acknowledge the assistance of Jennifer Obernier, who worked on the report until she left ILAR in August 2006, and of Lida Anestidou, who assumed this project upon her arrival at the National Academies in November 2006. Their work made this report possible.

Peter A. Ward, *Chair*
Committee on Recognition and Alleviation
of Distress in Laboratory Animals

Contents

Summary

This report is the first of two reports prepared as an update to the 1992 National Research Council (NRC) report *Recognition and Alleviation of Pain and Distress in Laboratory Animals*. In the 15 years since the first NRC publication on this subject, there has been considerable scientific progress in the areas of animal welfare and behavior, including attention to the subjects of stress and distress. U.S. regulations promulgated by the Animal Welfare Act and Public Health Service Policy as well as standards and practices promoted by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and the *Guide for the Care and Use of Laboratory Animals* (NRC 1996) mandate that pain and distress in laboratory animals be minimized or eliminated, except when scientifically justified. These policies address pain and distress jointly because both are considered unpleasant and potentially harmful to the animal subjects. From a scientific perspective, however, pain and distress are quite different and should be examined separately so that each receives appropriate emphasis. This is especially true for distress, which has historically been difficult to define and on which there has been relatively little research.

SCOPE OF THE STUDY

Due to both the paucity of information and the lack of a clear, widely accepted definition for distress, the scientific community using animals in research, including investigators, veterinarians, animal care staff, and animal care and use committees, has not had reliable guidance in recognizing,

assessing, or alleviating distress. Because minimization or elimination of distress experienced by laboratory animals is not only a regulatory requirement but also a moral obligation, it is imperative to attempt an evaluation of the state of the science and to translate current scientific knowledge into practical guidelines for use in laboratory animal facilities. Specifically, the Committee was tasked with preparing

> *a report on stress and distress [that] will review the current scientific literature regarding mechanisms of stress and distress for animal models used in biomedical research as well as the literature regarding methods for recognizing and alleviating distress. Emphasis will be placed on: the scientific understanding of causes and functions of stress and distress; determining when stress becomes distress; and identifying principles for recognition and alleviation of distress. Specific emphasis will be placed on the identification of humane endpoints in situations of distress and principles for minimizing distress in laboratory animals. While all possible scenarios cannot be included in this document, general guidelines and examples will be given to aid Institutional Animal Care and Use Committee (IACUC) members, investigators and animal care staff in making decisions about protocols using laboratory animals under current federal regulations and policies. Recommendations will be based on the most current scientific data where such data are available. The Committee will also identify gaps in the scientific literature where additional research data are needed.*

The Committee approached its task from the perspective of performance standards without describing—among others—factors such as intensity, duration, or types of perturbations, in part because this is an advisory document about an insufficiently understood phenomenon, but also because the Committee members believe that—within the current state of science—the best approach to recognize and alleviate distress is through best practices and professional judgment.

STRESS VERSUS DISTRESS

Various views, definitions, and language have been used in the discussion of *stress* and *distress*. Current scientific knowledge supports the concept that *stress* is a real or perceived perturbation to an organism's physiological homeostasis or psychological well-being. In its *stress response,* the body uses behavioral or physiological mechanisms to counter the perturbation. Events that precipitate *stress* (called *stressors*) can elicit any of a number of coping mechanisms or adaptive changes, including behavioral reactions, activation of the sympathetic nervous system and adrenal medulla, secretion of stress hormones (e.g., glucocorticoids and prolactin), and mobilization of the immune system.

Both stress and distress are meaningful terms that describe a state of being. While the biological responses to stress are better understood, the scientific, regulatory, and animal welfare communities disagree with respect to a universally accepted definition of distress. Although most definitions of *distress* characterize it as an aversive, negative state in which coping and adaptation processes in response to stressors fail to return an organism to physiological and/or psychological homeostasis, philosophical differences center on the inclusion of emotions and feelings affected by this state of being. Similarly, while it is accepted that failure of the organism to return to homeostasis adversely impacts an animal's well-being and leads to poor welfare, defining *well-being* without relying on some form of anthropomorphic measures is a challenge. Scientific research does not yet support objective criteria or principles with which to qualify distress, objective scientific assessment of subjective emotional states cannot be made, and while there is often a measure of agreement on the interpretation of physiologic and/or behavioral variables as indicators of stress, distress, or welfare status, there is not always a direct link. Further, the Committee postulates that even if a universally accepted definition existed, it could not be applied across all species and all conditions, because of the differential impact of the strain, age, gender, genetic background, and environment.

The transition to distress, which occurs when the body cannot cope against the assault of one or more stressors, depends on several factors. Of clear importance are stressor duration, stressor intensity, and the capacity of the individual animal to respond; changes in any of these increases the likelihood of behavioral or physical signs of distress. Thus, minor perturbations may be stressful and/or negatively affect an animal's moment-to-moment emotional state but they would not impair its adaptive capacity and therefore not cause distress (this may be unrelated to the state of the animal's welfare as illustrated in Figure 2-2). In contrast, a major homeostatic disruption (e.g., postsurgical infection), which causes measurable behavioral (e.g., withdrawal) and physiological (e.g., fever) changes that impair an animal's adaptive capacity, would be considered distressful and indicative of poor welfare. However, distress may not manifest itself with recognizable "maladaptive behaviors, such as abnormal feeding or aggression" (NRC 2003a, page 16) but instead begin with subclinical pathological changes (e.g., hypertension or immunosuppression) that can lead to overt disease. These physiological concepts should be integrated within and evaluated in concert with animal welfare principles.

RECOGNITION AND ASSESSMENT OF STRESS AND DISTRESS

While there are some specific behavioral measures of stress, relatively little is known about behavioral correlates of stress (i.e., behavioral changes

directly attributed to the presence of stress), and even less about those of distress. Thus, recognizing stress and distress in laboratory animals based on behavioral changes remains a significant challenge to investigators and animal care staff. A first-order approach to this challenge is to understand the animals' normal behaviors, while keeping in mind that such behaviors are neither invariant nor universal. Although normal behaviors may sometimes be characterized simply by a lack of atypical behavior, such as stereotypic (i.e., repetitive) or self-injurious behavior, some species and strain differences are not always easy to discern, and further complications are introduced by gender, age, physiological state, genetics, and genetic modification of the animals. Furthermore, it is not possible to recreate the full range of species-specific behaviors in the laboratory setting, as some types of behavior (e.g., severe aggression) are clearly undesirable from a management perspective.

Physiological effects of stress are mediated through the endocrine, neural, and immune systems and changes in stress hormone levels such as cortisol as well as the actions of the autonomic nervous system in response to known stressors have been well documented. However, research has not necessarily focused on deciphering these complex mechanisms in situations of suspected distress.

Assessment for the presence of stress should consider conditions that reliably produce it (e.g., exposure to a predator) and may be based on clinical and biochemical parameters such as activation of the hypothalamic-pituitary-adrenal (HPA) axis, changes in other hormones (e.g., prolactin), and changes in blood pressure and heart rate, and behavioral measures. An effective assessment of distress is predicated upon solid knowledge of physiologic behavior for each species and careful observation. It should integrate information from multiple behavioral and physiological parameters and should involve a team approach that includes researchers, veterinarians, and animal caretakers/technicians, as distress levels will vary in relation to the species, husbandry conditions, and experimental protocol as well as with each individual animal. The Committee points out that although the differentiation between abnormal behaviors associated with or caused by stress/distress and those observed in disease states (for example, both distressed and sick animals may not clean themselves and have matted fur coat) may be conceptually difficult, poor health means poor welfare. It is the Committee's opinion that, until more research is available, validated practices seeking what is best for the animals while maintaining the integrity of research protocols (i.e., the use of performance standards) should be used.

AVOIDING, MINIMIZING, AND ALLEVIATING DISTRESS

Efforts to avoid or minimize distress should follow the principles of the *Three Rs:* **refine**, **reduce**, and **replace**, which apply to daily husbandry as well as experimental procedures. Because most laboratory animals live outside normal habitats, they should, to the extent possible in an artificial environment, have the opportunity to express species-specific behaviors. Animal welfare evaluations should consider conditions of housing, husbandry, enrichment, and socialization. The Committee's philosophy has been to motivate investigators, veterinarians, and Institutional Animal Care and Use Committees (IACUCs) to embrace the *Three Rs* and through those criteria to act in the best interest of the animals while safeguarding the integrity of the research process.

Consideration of humane endpoints should be part of the experimental protocol in order to minimize or avoid subjecting an animal to adverse conditions. Pilot studies can be an effective option (for example in protocols known or anticipated to elicit distress, in dose-response or LD_{50} studies), while sound experimental design and statistical analysis are essential to ensure the use of appropriate number of animals. New minimally or non-invasive technologies that allow sophisticated tracking of disease progression, allow for reduction in animal numbers and/or earlier termination of experiments, thus avoiding prolonged and/or unnecessary discomfort to the animals. To address situations of unanticipated distress, the investigator, veterinary staff, and animal care personnel, working as a team and in compliance with the current regulations, should establish a plan to alleviate the distress, for example by removing an animal from the study, or through pharmacological treatment with anxiolytics, antidepressants, or neuroleptics.

The study of distress itself is important for both human and animal health. However, investigators who engage in research on distress using laboratory animal models, should, in consultation with the veterinarian and the IACUC, develop a plan that establishes limits to the levels of distress allowed in the experimental protocol. Appropriate methods to refine distress-related experimental designs include taking steps to alleviate distress after completion of the procedures or upon attainment of the research aims (e.g., maximum allowable weight loss as a percentage of normal body weight). As new methodologies and/or data from these studies become available, current practices in addressing stress and distress should be evaluated and modified accordingly.

FUTURE STUDIES AND RECOMMENDATIONS

Many questions in the field of laboratory animal distress remain unanswered. The Committee, therefore, offers the following suggestions for research directions that can improve our understanding of distress:

- determine whether there are biomarkers of distress that may be easily measured;
- use genomic and proteomic technologies to study the physiology and pathophysiology of stress and distress;
- develop possible distress predictors to be used as outcomes scores (i.e., to predict severity in clinical outcomes, mortality, etc., and adopt humane or surrogate endpoints) for laboratory animals, similar to the predictive severity scoring system used in human intensive care units;
- delineate the mechanisms of possible associations between stress/distress and disease behaviors or abnormal behaviors (e.g., stereotypies);
- study the influence of an organism's characteristics (e.g., gender, age, or genetic makeup) on the development of distress;
- identify refinements in euthanasia methods;
- study the potential use of historical controls in appropriate research protocols;
- determine parameters for optimal husbandry conditions for laboratory animals; and
- determine the appropriateness of experimental designs currently used for human research in studies that depend on laboratory animal models.

The Committee also provides the following recommendations:

1. **The *Three Rs* (refinement, reduction, and replacement) should be the standard for identifying, modifying, avoiding, and minimizing most causes of distress in laboratory animals.** While research on distress and methods of alleviating distress (e.g., the development of anesthesia or analgesia) may unavoidably cause animal suffering, the optimum goal of research and veterinary teams should be to reduce and alleviate distress in laboratory animals to the minimum necessary to achieve the scientific objective.

2. **Protocols should include efforts to improve housing and husbandry conditions through the judicious employment of strategies for enrichment, animal training, and socialization.** Well-trained,

competent, and attentive research and animal care personnel are crucial in providing relief from unintended distress that originates from the care and use of laboratory animals.

3. **Institutional support for and embrace of a commitment to animal welfare of the laboratory animals is essential. Veterinarians and animal care personnel who work with research animals on a daily basis should have adequate time and contact with the animals to properly evaluate their well-being.** Funding for training programs is crucial to the training and development of specialized laboratory animal veterinarians and animal behaviorists and should increase, because in addition to such objective measurements as weight loss or lack of grooming, clinical judgment is vital to effective assessments of stress and distress.

4. **Appropriate statistical methodologies are an essential tool for the avoidance, minimization, and alleviation of distress.**

5. **There should be a clearinghouse (or some other venue such as a website or a specialized peer-reviewed journal) for publication of research on the effects of enrichment strategies on parameters such as physiology, distress, and endpoints for all laboratory animals** (one useful example is the Primate Enrichment Database hosted by the Animal Welfare Institute).[1] Although a variety of journals (such as *Lab Animal, Applied Animal Behaviour Science, Animal Welfare, Laboratory Animals, Contemporary Topics in Laboratory Animal Science, Comparative Medicine*) publish research pertaining to animal welfare, the highly specialized nature of the field makes it difficult for the larger scientific community to remain informed about recent advances and ongoing debates. Peer-reviewed biomedical research journals should be more open to submissions from scientists whose research focuses on animal welfare issues so that concerns about research interference or unjustified expenses can be debated on scientific, ethical, or regulatory grounds.

6. Obtaining funding for welfare research is often difficult, especially when project applications compete against other fields of science due to lack of an appropriate/separate research oversight body. In the United Kingdom the funds available for welfare research have increased dramatically with the founding of the National Center for the Replacement, Refinement and Reduction of Animals in

[1] http://www.awionline.org/SearchResultsSite/enrich.aspx.

Research (NC3Rs).[2] In the United States, the National Institutes of Health, Environmental Protection Agency, and other federal institutions have occasionally provided funding to develop or validate nonanimal or nonvertebrate alternatives. Funding for laboratory animal welfare research, however, is usually available only in small amounts from nongovernmental organizations such as the Animal Welfare Institute, the Johns Hopkins Center for Alternatives to Animal Testing, the American College of Laboratory Animal Medicine, and the American Association for Laboratory Animal Science. **Given the impact of better animal welfare on science as well as the growing public interest in the treatment of laboratory animals, federal agencies and large foundations that support biomedical and behavioral research should make funds available specifically for the avenues of investigation listed above and for other related topics.**

7. **Animal welfare scientists and researchers and scientists who use animal models should communicate with each other more frequently in order to compare objectives and progress and to identify opportunities for collaboration.** Neutral groups and/or other established research and science policy entities can provide platforms and venues for such exchanges.

REFERENCE

NRC (National Research Council). 2003a. *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research*. Washington, DC: The National Academies Press.

[2]NC3Rs website: www.nc3rs.org.uk.

1

Introduction

In 1992, the National Research Council published a report titled *Recognition and Alleviation of Pain and Distress in Laboratory Animals* "to help scientists, research administrators, institutional animals care and use committees, and animal care staff to address the difficult questions of the presence and alleviation of animal pain and distress" (NRC 1992, p. 1). The need for assistance in this area has persisted, and, with the advent of new scientific discoveries, the generation of genetically modified animals, and continued regulatory emphasis on minimizing pain and distress in laboratory animals, it became evident that the 1992 report had become outdated. The Institute for Laboratory Animal Research (ILAR) received several requests from the veterinary and biomedical communities to convene a Committee to update the report. After many discussions with constituents and several sponsors, the National Academies opted to update the 1992 report as two separate reports, one on distress and one on pain, because although they are linked in regulation, they are quite different scientifically.

REGULATIONS GOVERNING PAIN AND DISTRESS IN LABORATORY ANIMALS

Public concern for laboratory animals focuses on their pain and distress, and so, although a majority of the public supports the use of animals in biomedical research, that support diminishes when the animals are subjected to pain and/or distress. In response to these views government policies and laws mandate the minimization or elimination of pain and distress. For example, according to U.S. Government Principle IV for the

Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training, "Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals" (IRAC 1985). Similarly, the federal Animal Welfare Act Regulations (USDA 2005), the *Guide for the Care and Use of Laboratory Animals* (NRC 1996), U.S. Public Health Service Policy for the Humane Care and Use of Animals (DHHS 2002), and policies of the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC International) all require the identification, minimization, and elimination of sources of pain and distress in laboratory animals, consistent with the goals of the research.

These policies address pain and distress jointly as both are considered unpleasant and potentially harmful to the animal subjects. From a scientific perspective, however, pain and distress are quite different and should be examined separately so that each receives appropriate emphasis. This is especially true for distress, as it has been difficult to define and there is relatively little research in this area. In fact, only a small portion of the 1992 report discussed distress because at that time very little scientific information was available. While more information was available for this report, it is still difficult to pinpoint exact measures of distress.

Due to the paucity of information and the lack of a clear, widely accepted definition for distress, the biomedical research community, including investigators, veterinarians, animal care staff, and IACUCs, has not had reliable guidance in recognizing, assessing, or alleviating distress. Because regulations call for the minimization or elimination of distress, it is imperative to attempt an evaluation of the state of the science and to translate current scientific knowledge into practical guidelines for use in laboratory animal facilities. Specifically, the Committee was tasked with preparing

> *a report on stress and distress [that] will review the current scientific literature regarding mechanisms of stress and distress for animal models used in biomedical research as well as the literature regarding methods for recognizing and alleviating distress. Emphasis will be placed on: the scientific understanding of causes and functions of stress and distress; determining when stress becomes distress; and identifying principles for recognition and alleviation of distress. Specific emphasis will be placed on the identification of humane endpoints in situations of distress and principles for minimizing distress in laboratory animals. . . . [G]eneral guidelines and examples will be given to aid IACUC members, investigators and animal care staff in making decisions about protocols using laboratory animals under current federal regulations and policies. Recommendations will be*

based on the most current scientific data where such data are available. The Committee will also identify gaps in the scientific literature where additional research data are needed.

ORGANIZATION OF THE REPORT

This report is the result of the Committee's work to address this charge, and it is organized as follows. In the common vernacular, stress and distress are used almost interchangeably without consideration of the cause or scientific implications. However, the Committee attempted to the best of its ability to make a clear distinction between the scientific concepts of stress and distress, noting, in particular, that the causes and consequences of the latter are less well defined. Although a distinct definition of distress was not produced, Chapter 2 presents a balanced discussion of stress and distress that incorporates animal welfare perspectives based on the members' best professional judgment. The report embraces the idea (also reflected in the U.S. Government Principles) that pain and distress are clearly two different things. Distress is caused by more than momentary painful situations (both acute and chronic), while non-pain-related distress exists as well. In fact, the latter, given the insidiousness of its causes, may be even more prevalent, as painful insults are easier to recognize and deal with than, for example, inadequate husbandry conditions.

In addition to compiling the most up-to-date information on the physiology of stress and distress, the Committee in Chapter 3 used its expertise in the area of animal behavior to provide the most current scientifically based information on normal and abnormal behaviors of some of the most commonly used laboratory species. While this information is not exhaustive, it does include pertinent examples of situations in which laboratory animals may experience distress. An important point of this chapter is that in order to recognize distress in animals, it is necessary to know their normal individual behaviors. Additional information on behaviors and score sheets for several commonly used laboratory species are included in the Appendix.

One way to minimize or eliminate distress, however, is to avoid it altogether. Chapter 4 outlines current practices and highlights specific issues to be considered for alleviating, minimizing, or preventing distress. Some measures for distress prevention include optimization of housing, enrichment, and socialization conditions based on the needs of the individual species or strains being used. Although much general information is available about acceptable conditions for maintaining animals in laboratories, in many instances scientific evidence is minimal or lacking. In those cases it is necessary to rely on the expert opinion of the professionals who work directly with the animals on a regular basis. In addition, research protocols for studies in which animal distress is anticipated should consider humane

endpoints as well as the use of appropriate statistics and experimental design to minimize the number of animals that must be used. Investigators should establish a partnership with the veterinarians and animal care staff and make provisions in cases of either anticipated or unanticipated distress.

The intent of this report is to assist investigators, veterinarians, animal care staff, and IACUCs in understanding distress so that it can be recognized, alleviated, or prevented. The Committee urges readers to consider the information in this report very carefully and to exercise professional judgment in evaluating situations where distress occurs or is likely to occur. As new information becomes available, it should be incorporated into practice and decision making in the care of laboratory animals. The Committee hopes that this report will be useful in assisting readers to comply with regulations, to achieve reliable scientific outcomes, and, especially, to provide the best possible care for their animal subjects.

REFERENCES

DHHS (Department of Health and Human Services), National Institutes of Health, Office of Laboratory Animal Welfare. 2002. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Available at: http://grants.nih.gov/grants/olaw/references/phspol.htm. Accessed January 30, 2008.

IRAC (Interagency Research Animal Committee). 1985. The U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Federal Register Vol. 50, No. 97 (May 20, 1985). Office of Science and Technology Policy. Available at: http://grants.nih.gov/grants/olaw/references/phspol.htm#USGovPrinciples. Accessed January 30, 2008.

NRC (National Research Council). 1992. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

NRC. 1996. *Recognition and Alleviation of Pain and Distress in Laboratory Animals*. Washington, DC: National Academy Press.

USDA (U.S. Department of Agriculture). 2005. 9CFR2.31. Code of Federal Regulations, title 9, Volume 1, part 2, subpart C, section 2.31 (d) (1) (i): Institutional Animal Care and Use Committee. Available at: http://www.aphis.usda.gov/animal_welfare/downloads/awr/9cfr2.31.txt. Accessed January 30, 2008.

2

Stress and Distress: Definitions

INTRODUCTION

The various views and language used in the discussion of *stress* and *distress* lead to confusion in the scientific, regulatory, and animal welfare communities. Indeed, the animal welfare literature itself does not distinguish *stress* from *distress* in any systematic fashion, and the term *distress* rarely appears in the biomedical sciences literature. In addition, the U.S. Government Principles and the Animal Welfare Act (see below) use both terms without definitions. Moreover, the general public often uses "stress" and "distress" interchangeably, and frequently in conjunction with the term "suffering," thus blurring distinctions between these concepts. Because there is in fact good scientific evidence for both an adaptive *stress* response and a state of *distress*, it is important to distinguish these terms. Even though this chapter attempts to clarify these terms as much as possible, the available scientific information—while useful—is far from complete, and distress remains a complex and still poorly understood phenomenon. This chapter, therefore, is an amalgam of current scientific information, along with the Committee members' perspectives, best professional judgment, and expert opinion.

While there is general agreement that pain and distress usually have a direct impact on animal welfare and quality of life, the descriptions of these conditions have evolved from different views and terminologies. The U.S. Animal Welfare Act (AWA 1990) uses the words "pain and distress", whereas in the European Union's Directive on the Protection of Animals Used for Experimental and Other Scientific Purposes (EEC 1986) the equiva-

lent phrase is "pain, suffering, distress and lasting harm." Distress can be used to describe a state in which an animal, unable to adapt to one or more stressors, is no longer successfully coping with its environment and its well-being is compromised.

Generally, a state of distress develops over a relatively long period of time; however, short, intense stressor(s) can also compromise animal well-being and induce acute distress. Thus, an animal may be in distress even if it appears to recover rapidly after the removal of the stressor or the conclusion of the procedure.

WHAT IS STRESS?

Stress is an inferred internal state. Because no single biological parameter can adequately inform on a stressful condition and no single stress response is present in all stress-related situations, there are many definitions of stress based primarily on metrics used to test hypothetical models of this state. A general distillation of the literature suggests that stress denotes a real or perceived perturbation to an organism's physiological homeostasis or psychological well-being. In its stress response the body uses a constellation of behavioral or physiological mechanisms to counter the perturbation and return to normalcy. Events that precipitate stress (called stressors) elicit any of a number of coping mechanisms or adaptive changes, including behavioral reactions, activation of the sympathetic nervous system and adrenal medulla, secretion of stress hormones (e.g., glucocorticoids and prolactin), and mobilization of the immune system. Stress responses may involve at least one and perhaps several of the above systems, although none of them is by itself necessary or sufficient to denote stress. Furthermore, the absence or presence of any of these responses does not include or preclude the identification of a stressful state (for a comprehensive review see Moberg 2000). Stress responses have several key attributes:

- They serve to promote physiological and psychological adaptation and are, therefore, beneficial and desirable. For example, activation of the sympathoadrenomedullary (SAM) system rapidly increases blood flow to the musculature and raises circulating glucose levels, resulting in an enhanced capacity to flee or fight (the "fight or flight" response). Over a longer time frame, glucocorticoid production in response to infection helps restrict the immune system, thus preventing deleterious effects of inflammatory factors on tissues (Gillis et al. 1979; Munck et al. 1984).
- Apparent stress reactions can occur in situations unrelated to stress, and therefore their presence alone is not sufficient to indicate stress. For example, the diurnal rhythm of glucocorticoid secretion in most

animals results in glucocorticoid levels at the diurnal peak that can rival those measured following stressor exposure (Dallman et al. 1987). Thus, no single parameter can serve as a litmus test for stress and diagnosis of stress based on a single metric can be misleading.

- Stressors may not necessarily be unpleasant (defined by the animal's willingness to terminate the stressor); they can be pleasurable (Selye's "eustress" concept; Selye 1975), as defined by an organism's willingness to obtain the stressor. For example, naturally rewarding behaviors, such as exercise, increase sympathetic activity and circulating glucocorticoids in a profile very similar to that seen following aversive stressors (Droste et al. 2003). The self-administration of a drug, such as cocaine, similarly fits the definition of a stressor because multiple physiological systems are recruited to adapt to and oppose the drug's action.
- Physiological and behavioral responses are stressor-specific and so the processes engaged to restore homeostasis or well-being also differ. Thus, the following are all considered stressors, although they elicit variable behavioral and physiologic responses: viral or bacterial infection, threat of physical harm, drugs, exercise, sexual activity, high altitude, restraint, hunger, and thirst. Many of the above elicit "useful" or "good" stress, which is beneficial to the animal in the long term. For example, while caloric restriction might be stressful or unpleasant because chronic hunger is involved, it promotes longevity and good health (Kemnitz et al. 1994; Lawler et al. 2005; Messaoudi et al. 2006).
- Responses to stressors are variable due both to individual (some individuals are better able to cope than others) and intraspecies differences. For example, strain differences in inbred mice may result in dramatically different physiological or behavioral responses to stress[1] (Crawley 2000; Hedrick and Bullock 2004; Silver 1995).

WHAT IS DISTRESS?

Distress has many definitions (see, for example, various dictionaries). Most definitions characterize distress as an aversive, negative state in which coping and adaptation processes fail to return an organism to physiological and/or psychological homeostasis (Carstens and Moberg 2000; Moberg 1987; NRC 1992). Progression into the maladaptive state may be due to a severe or prolonged stressor or multiple cumulative stressful insults with

[1]Detailed information on behavioral and physiological data of various subsets of murine inbred strains is available at the Mouse Phenome Database at the Jackson Laboratory; http://aretha.jax.org/pub-cgi/phenome/mpdcgi.

deleterious effects on the animal's welfare. Distress can follow both acute and chronic stress, provided that the body's biological functions are sufficiently altered and its coping mechanisms overwhelmed (Moberg 2000).

The transition of stress to distress depends on several factors. Of clear importance are stressor duration and intensity, either of which is likely to produce behavioral or physical signs of distress. For example, short-term restraint does not cause marked problems in adaptation, whereas prolonged restraint can result in behavioral or physiological distress sometimes expressed by vocalization or gastric ulcers (Ushijima et al. 1985). In addition, predictability and controllability (i.e., the ability of the animal to control its environment) are important determinants in the transition of stress to distress. Numerous studies indicate that, in animals that can predict the onset of a stressful stimulus or control its duration, the behavioral and physiological impacts of stressor exposure are attenuated. Notable among these studies are findings that rats exposed to inescapable shock develop clear signs of distress, whereas yoked rats that can terminate shock exposure do not, despite subjection to the same intensity and duration of shock experience (Maier and Watkins 2005).

Furthermore, the stress response may induce insufficient or inappropriate changes in the behavioral and physiologic control systems (noted above) or inadequate or undesirable responses to their output signals. For example, chronic social subordination has been shown to elicit behavioral withdrawal, prolonged alterations in the hypothalamic-pituitary-adrenal (HPA) axis output, and subsequent immunosuppression (Blanchard et al. 2001), all of which preclude effective coping and adaptation. Further studies have shown that in chronic distress states, such as depression, the glucocorticoid feedback systems fail (Carroll et al. 1976). Thus, if stress responses themselves fail to appropriately cope or produce successful adaptation they may be not merely ineffective but actively deleterious. For example, while corticosteroid responses are essential for the adaptation process, marked or prolonged hypersecretion can produce pronounced metabolic and immune dysfunction (Munck et al. 1984).

Should an animal have the option to behaviorally express a choice in response to a stressful condition and thus exercise some control over its environment, then its adaptive behaviors should be distinguished from maladaptive ones displayed in distress (NRC 2003a, page 22; Mench 1998). However, a cause and effect relationship between various abnormal behaviors and distress or the operationalization and validation of the degree of abnormality associated with distressed states has not yet been established. Distress may not always manifest itself with recognizable "maladaptive behaviors, such as abnormal feeding or aggression" (NRC 2003a, page 16) but instead with subclinical pathological changes, such as hypertension and immunosuppression, which are not behaviorally identifiable. As Moberg

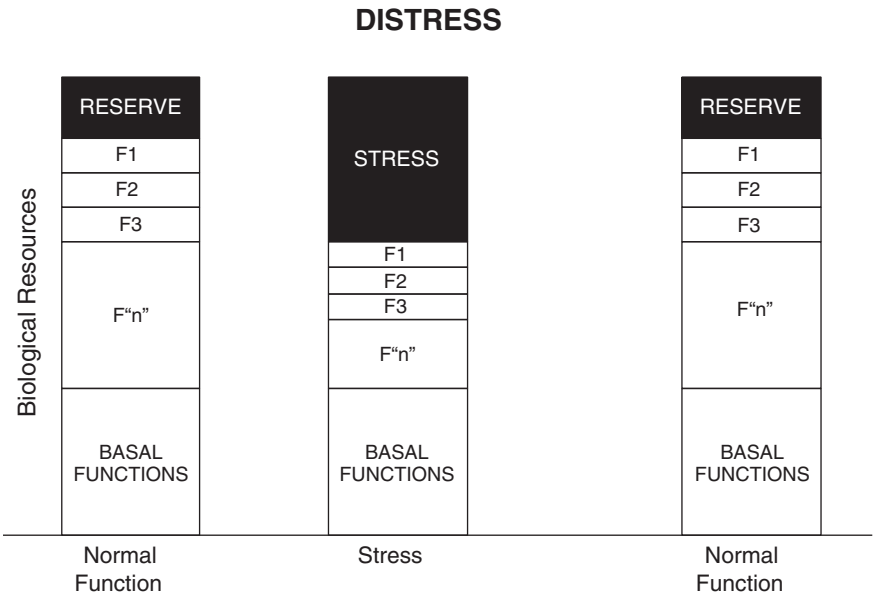


FIGURE 2-1 Reprinted with permission from Macmillan Publishers Ltd: [Lab Animal] (Moberg 1999), copyright (1999). Prolonged or severe stress depletes bodily reserves and affects normal functions thus requiring extended time to revert to homeostasis. During the recovery period, the animal's well-being and welfare are compromised and the period of distress will last until the biological resources are sufficiently replenished (Moberg 2000). The shift in biological resources, such as stunted growth in distressed young animals (Moberg 1999), or evidence of maladaptive behaviors (NRC 1992) that occur in this general scheme of transition to and establishment of distress could be useful in recognizing distress (NRC 2003a, page 21).

proposed in his 1999 paper "When Does Stress Become Distress", the use of reserve resources to cope with prolonged or severe stress has a negative impact on other bodily functions (including behavior) and leads to distress. In the hypothetical scheme depicted in Figure 2-1, the "biological cost of distress" requires a prolonged recovery period to revert to homeostasis (Carstens and Moberg 2000).

IMPLICATIONS FOR ANIMAL WELFARE

Current understanding of animal welfare as a measure of the animals' quality of life exists in the context of the social and cultural history of animal care and use as well as an expanding knowledge base related to

animal physiology and ethology. As early as 1964 the Brambell Committee acknowledged that "welfare is a wide term that embraces both the physical and mental well being of the animal". The authors further elaborated that evaluations of animal welfare must take into account the scientific evidence derived from the animals' structure, functions, and behavior (Brambell 1965; Duncan 2005). Although clinical signs can be used to assess physical well-being, and behavioral studies can provide information about animals' preferences and cognitive state (for a review of validated animal models for fear and depression see Phelps and LeDoux 2005; also see Bateson and Matheson 2007), the Committee would like to emphasize that no physiologic measures exist to date with which to assess mental well-being directly. Nevertheless, discussions about animal welfare in the laboratory as well as in farm animal communities take into consideration a variety of criteria to assess an animal's quality of life. It has been proposed that the most important consideration for the assessment of an animal's welfare is its emotional state (Duncan 2005). Be that as it may, some of these criteria focus on the animals' ability to experience pleasure and pain (as defined in Bentham 1879), or their higher cognitive capacities (Nuffield Council on Bioethics 2005), while others consider the animals' housing and husbandry conditions. The latter are of course easier to define and to assess, and are therefore the focus of more scientific research and literature.

Housing and husbandry conditions should permit an animal to be physically healthy (i.e., not interfere with its biological functioning), live a natural life, behave more or less normally, and be free of pain and other negative circumstances (that induce negative affective states; Fraser et al. 1997).[2] Concerns for animal welfare are often focused on what the animal may experience (Kirkwood 2007), including its ability to control its environment or predict the onset of a stressor. In these discussions, the term "suffer/suffering" is often used, albeit controversially due to lack of consensus with respect to the adverse emotional states to which it may allude, such as pain, distress, boredom, deprivation, fear, frustration, and grief, in which an animal may be said to suffer even for only a few minutes.[3]

Descriptors of an animal's welfare are qualitative and range from "poor" to "good" (other adjectives commonly used include "negative", "compromised", "neutral", and "positive"). Welfare may be compromised briefly (e.g., during handling, injection, or exposure to a predator) or over longer periods of time (e.g., in the solitary housing of a social species, or in the

[2]For additional discussion of what is normal or natural with regard to laboratory animals, see Chapters 3 and 4.

[3]The Veterinarian's Oath outlines the moral obligation toward the alleviation of animal suffering by stating that ". . . I solemnly swear to use my scientific knowledge and skills for the benefit of society through . . . [in part] the relief of animal suffering. . . ." (AVMA, http://www.avma.org/issues/animal_welfare/).

provision of housing without appropriate enrichment).[4] In order to prevent poor or deteriorating welfare, researchers, animal care staff, and institutions have a responsibility to provide high-quality care for laboratory animals, including ready access to fresh water and a nutritive diet; an environment that ensures shelter and comfort; prevention as well as rapid diagnosis and alleviation (as appropriate) of pain, injury, and disease; species-appropriate space, facilities, and (if appropriate) companionship; and conditions and treatment that do not cause negative emotional states. Fraser and colleagues suggest that good animal welfare implies the absence of pain, fear, and hunger; enables a high level of biological functioning (i.e., normal growth, freedom from disease); and (more controversially) enables animals to experience positive emotional experiences such as comfort and contentment (Fraser et al. 1997).

It is possible for an animal to be in a state of poor health that does not impinge on its welfare or emotional state and that may even last for some time without the animal's conscious awareness. For example, an animal might have a life-threatening aneurysm but be unaware of it and therefore not experience a negative emotional state. In the longer term, however, a breakdown in an animal's ability to cope with its environment is likely to lead to adverse emotional states and poor welfare. Some of these cases may be quite minor and not give rise to significant ethical concerns; but prolonged or intense circumstances would compromise the animal's welfare enough to warrant concern and also significantly affect the research results.

An attempt to graphically depict the relationship between distress and welfare is shown in Figure 2-2. Whereas minor perturbations (e.g., short-term restraint of a rodent) affect an animal's welfare in terms of its moment-to-moment emotional state, they do not impair its adaptive capacity and thus do not cause distress. In contrast, a major homeostatic disruption (e.g., postsurgical infection), which causes measurable behavioral (withdrawal) and physiological (fever) changes that impair the adaptive capacity of the animal, is considered "distressful" and is indicative of "poor welfare".

Onset of distress can be difficult to recognize. A safe assumption is to follow the fourth principle of the U.S. Government Principles for Utilization and Care of Vertebrate Animals used in Testing, Research and Teaching: "Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings

[4]For more information on the effects of housing on brain function or enrichment see Chapter 3. Additional information is contained in articles by the behaviorists Joseph Garner, Hanno Würbel, and Georgia Mason.

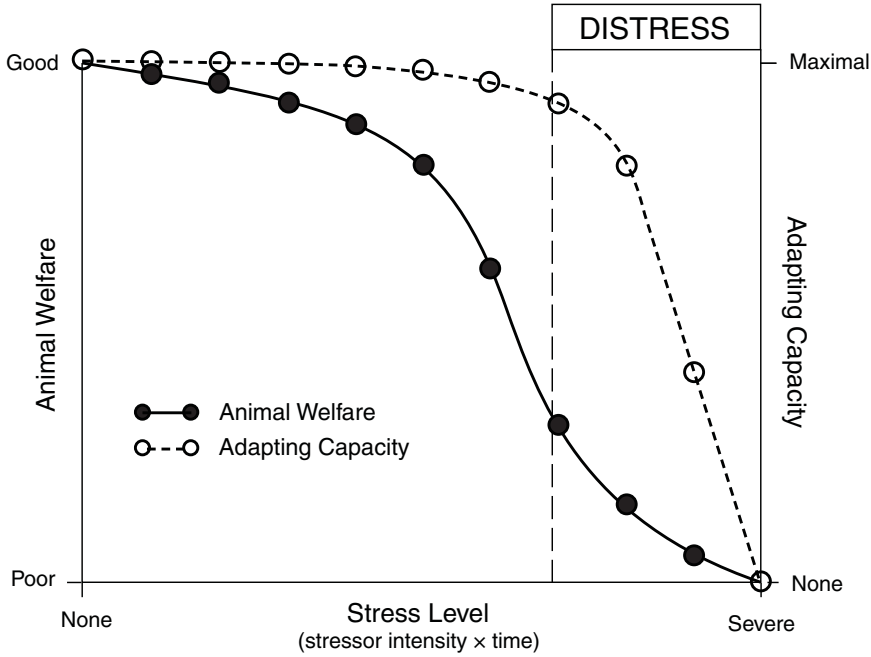


FIGURE 2-2 Hypothetical depiction of the relationship of stress, distress, adaptive capacity, and animal welfare. An animal's quality of life may be progressively deteriorating while it is still successfully coping with a stressor. The precise moment of transition to the maladaptive state or what precipitates it is unknown, but once the tipping point is attained, the deterioration into a severely sick or debilitated animal occurs fairly quickly. At this point, welfare conditions are very poor and immediate ameliorative action is necessary.

may cause pain or distress in other animals" (IRAC 1985). A degree of critical anthropomorphism, outlined above and in the writings of Morton and colleagues (Morton et al. 1990), coupled with behavioral assessments will likely provide the most direct understanding of an animal's response to a stressor. Useful indicators include the animal's choice to continue or stop feeding while in a stressful situation, choice tests that demonstrate how (non)aversive a particular stressor is, or demand studies that titrate the extent of the animal's attraction or aversion to a potential stressor. These gauges of avoidance or aversion may be complemented by physiological data measuring elevated hypothalamic-pituitary-adrenocortical axis (HPA) or sympathoadrenomedullary system (SAM) activity (gene or protein activation), elevated hormone levels, or increased activity in target organs (e.g.,

heart rate, blood pressure, glucose levels). Many authors have pointed to the desirability of using multiple measures to obtain a more comprehensive data set (Rushen 1991). It is important to underscore that reliance on a single measurement of stress may result in erroneous conclusions. Chapter 3 provides more details on distress recognition.

IMPLICATIONS FOR RESEARCH

There is a rich literature documenting the interference of stress in behavioral and/or physiological endpoints. Strong evidence in rodents has shown that mild stress of 2-3 months duration—a regimen that produces no signs of overt distress—alters the animals' performance in tests of anxiety, depression, and memory (D'Aquila et al. 1994; Rossler et al. 2000; Song et al. 2006; Willner 1997). Other findings indicate that rats' habituation to a test environment can dramatically affect their response to a toxic substance (Damon et al. 1986). On the other hand, in some cases (such as lower anxiety behavior in the elevated plus maze) the effects of stress may actually be beneficial to the experimental procedure, indicating that prolonged stress may not be uniformly detrimental. Chapter 3 documents the contamination of experimental data by unwanted or uncontrolled stress due to inadequate husbandry, noisy environments, olfactory stimuli, or other factors.

The impact of distress on both animal welfare and research results is likely even more pronounced than that of stress. Animals exposed to prolonged severe stress experience underlying changes in physiological functions (e.g., gastric lesions [Ushijima 1985] or immunosuppression [Tournier 2001]) that can interfere with experimental manipulations; alter experimental variables such as behavior (Morton and Griffiths 1985), drug dosing (Saranteas et al. 2004) and clearance; change the progress of a disease (Johnson et al. 2006); and contribute to morbidity and mortality. A variety of stressors can contribute to unintended distress, from postoperative pain or infection to barren housing conditions or the solitary confinement of an individual of a social species (Gunn and Morton 1995; Morton et al. 1993). Stereotypies, abnormal repetitive behaviors indicative of poor well-being (Garner et al. 2003) that are often observed in distressed animals, are thought to reflect defective brain function (Würbel 2001) and to be a result of poor animal welfare (Mason and Latham 2004). Stereotypies are thus likely to interfere with behavioral, neuroscience, and pharmacological studies.[5]

The impact of stress and distress on the quality of scientific research can result in the generation of compromised data, which in turn necessitates the use of more animals. This outcome is inconsistent with two of the

[5]It should be noted that the negative connotations of stereotypies are not universally accepted. For further discussion see Chapter 3.

Three Rs: reduction in the number of animals used in an experiment, and refinement of the protocol to minimize or eliminate distress for the animals used (Russell and Burch 1959).

CONCLUSIONS

In an effort to reduce the confusion surrounding the definitions of stress and distress and as a basic framework to inform future research in these areas, the Committee offers the following summary of distinctions between the two concepts:

- Stress and distress are dissociable concepts, distinguished by an animal's ability or inability to cope or adapt to changes in its immediate environment and experience.
- Stress responses are normal reactions to environmental or internal perturbations and can be considered adaptive in nature. Distress occurs when stress is severe, prolonged, or both.
- The concepts of stress and distress can be distinguished from that of welfare, in that an adaptive and beneficial stress response may occur against a backdrop of a transient negative emotional state.
- Both stress and distress represent potential complications in a wide range of experiments, and should be proactively addressed by good experimental design.

REFERENCES

AVMA (American Veterinary Medical Association). Issues in animal welfare. Available at www.avma.org/issues/animal_welfare. Accessed February 2, 2007.

AWA (Animal Welfare Act). 1990. Available at www.nal.usda.gov/awic/legislat/awa.htm. Accessed February 6, 2007.

Bateson, M. and S. M. Matheson. 2007. Performance on a categorisation task suggests that removal of environmental enrichment induces pessimism in captive European Starlings *(Sturnus vulgaris). Anim Welfare* 16(Suppl):33-36.

Bentham, J. 1879. *An Introduction to the Principles of Morals and Legislation*. 2005 ed. Boston: Adamant Media Corp.

Blanchard, R. J., C. R. McKittrick, and D. C. Blanchard. 2001. Animal models of social stress: Effects on behavior and brain neurochemical systems. *Physiol Behav* 73(3):261-271.

Brambell, F. W. R. 1965. Report of the Technical Committee to Enquire into the Welfare of Animals Kept Under Intensive Livestock Husbandry Systems. London: HMSO.

Carroll, B. J., G. C. Curtis, and J. Mendels. 1976. Neuroendocrine regulation in depression, I: Limbic system-adrenocortical dysfunction. *Arch Gen Psychiat* 33(9):1039-1044.

Carstens, E. and G. P. Moberg. 2000. Recognizing pain and distress in laboratory animals. *ILAR J* 41(2):62-71.

Crawley, J. N. 2000. *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*. New York: Wiley-Liss, John Wiley & Sons, Inc.

Dallman, M. F., S. F. Akana, C. S. Cascio, D. N. Darlington, L. Jacobson, and N. Levin. 1987. Regulation of ACTH secretion: Variations on a theme of B. *Recent Prog Horm Res* 43:113-173.

Damon, E. G., A. F. Eidson, C. H. Hobbs, and F. F. Hann. 1986. Effect of acclimation to caging on nephrotoxic response of rats to uranium. *Lab Anim Sci* 36(1):24-27.

D'Aquila, P. S., P. Brain, and P. Willner. 1994. Effects of chronic mild stress on performance in behavioural tests relevant to anxiety and depression. *Physiol Behav* 56(5):861-867.

Droste, S. K., A. Gesing, S. Ulbricht, M. B. Muller, A. C. Linthorst, and J. M. Reul. 2003. Effects of long-term voluntary exercise on the mouse hypothalamic-pituitary-adrenocortical axis. *Endocrinology* 144(7):3012-3023.

Duncan, I. J. H. 2005. Science-based assessment of animal welfare: Farm animals. *Rev Sci Tech OIE* 24(2):483-492.

EEC (European Economic Commission). 1986. Council Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes. Available at ec.europa.eu/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf. Accessed January 30, 2008.

Fraser, D., D. M. Weary, E. A. Pajor, and B. N. Milligan. 1997. A scientific conception of animal welfare that reflects ethical concerns. *Anim Welfare* 6(3):187-205.

Garner, J. P., C. L. Meehan, and J. A. Mench. 2003. Stereotypies in caged parrots, schizophrenia and autism: Evidence for a common mechanism. *Behav Brain Res* 145(1-2):125-134.

Gillis S., G. R. Crabtree, and K. A. Smith. 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J Immunol* 123(4):1624-1631.

Gunn, D. and D. B. Morton. 1995. Twenty-four hour study of the behavior of New Zealand White rabbits in cages. *Appl Anim Behav Sci* 45:277-292.

Hedrick, H. and G. Bullock, eds. 2004. *The Laboratory Mouse*. San Diego: Elsevier Academic Press.

IRAC (Interagency Research Animal Committee). 1985. The U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Federal Register Vol. 50, No. 97 (May 20, 1985) Office of Science and Technology Policy. Available at: http://grants.nih.gov/grants/olaw/references/phspol.htm#USGovPrinciples. Accessed January 30, 2008.

Johnson, R. R., T. W. Prentice, P. Bridegam, C. R. Young, A. J. Steelman, T. H. Welsh, C. J. Welsh, and M. W. Meagher. 2006. Social stress alters the severity and onset of the chronic phase of Theiler's virus infection. *J Neuroimmunol* 175(1-2):39-51.

Kemnitz, J. W., E. B. Roecker, R. Weindruch, D. F. Elson, S. T. Baum, and R. N. Bergman. 1994. Dietary restriction increases insulin sensitivity and lowers blood glucose in rhesus monkeys. *Am J Physiol-Endoc M* 266(4):E540–E547.

Kirkwood, J. K. 2007. Quality of life: The heart of the matter. *Anim Welfare* 16:3-7.

Lawler, D. F., R. H. Evans, B. T. Larson, E. L. Spitznagel, M. R. Ellersieck, and R. D. Kealy. 2005. Influence of lifetime food restriction on causes, time, and predictors of death in dogs. *J Amer Vet Med Assoc* 226:225-231.

Maier, S. F. and L. R. Watkins. 2005. Stressor controllability and learned helplessness: The roles of the dorsal raphe nucleus, serotonin, and corticotropin releasing hormone. *Neurosci Biobehav R* 29(4-5):829-841.

Mason, G. J. and N. R. Latham. 2004. Can't stop, won't stop: Is stereotypy a reliable animal welfare indicator? *Anim Welfare* 13:S57-S69.

Mench, J. A. 1998. Why it is important to understand animal behavior. *ILAR J* 39:20-26.

Messaoudi, I., J. Warner, M. Fischer, B. Park, B. Hill, J. Mattison, M. A. Lane, G. S. Roth, D. K. Ingram, L. J. Picker, D. C. Douek, M. Mori, and J. Nikolich-Zugich. 2006. Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates. *P Natl Acad Sci USA* 103(51):19448-19453.

Moberg, G. P. 1987. Problems in defining stress and distress in animals. *J Am Vet Med Assoc* 191(10):1207-1211.

Moberg, G. P. 1999. When does stress become distress? *Lab Anim* 28(4):422-426.

Moberg, G. P. 2000. Biological response to stress: Implications for animal welfare. In *The Biology of Animal Stress*, G. P. Moberg and J. A. Mench, eds. Wallingford, UK: CAB International. 1-21 pp.

Morton, D. B. and P. H. M. Griffiths. 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Record* 116(16):431-436.

Morton, D. B., G. M. Burghardt, and J. A. Smith. 1990. Critical anthropomorphism, animal suffering, and the ecological context. *Hastings Cent Rep* 20(3):S13-S19.

Morton, D. B., M. Jennings , G. R. Batchelor, D. Bell, L. Birke, K. Davies, J. R. Eveleigh, D. Gunn, M. Heath, B. Howard, P. Koder, J. Phillips, T. Poole, A. W. Sainsbury, G. Sales, D. J. A. Smith, M. Stauffacher, and R. J. Turner. 1993. Refinements in rabbit husbandry. *Lab Anim* 27(4):301-329.

Munck, A., P. M. Guyre, and N. J. Holbrook. 1984. Physiological functions of glucocorticoids in stress and their relations to pharmacological actions. *Endocr Rev* 5(1):25-44.

NRC (National Research Council). 1992. *Recognition and Alleviation of Pain and Distress in Laboratory Animals*. Washington, DC: National Academy Press.

NRC. 2003a. *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research*. Washington, DC: The National Academies Press.

Nuffield Council on Bioethics. 2005. *The Ethics of Research Involving Animals*. London: Nuffield Council on Bioethics.

Phelps, E. A. and J. E. LeDoux. 2005. Contributions of the amygdala to emotion processing: From animal models to human behavior. *Neuron* 48(2):175-187.

Rossler, A. S., C. Joubert, and G. Chapouthier. 2000. Chronic mild stress alleviates anxious behaviour in female mice in two situations. *Behav Process* 49(3):163-165.

Rushen, J. 1991. Problems associated with the interpretation of physiological data in the assessment of animal welfare. *Appl Anim Behav Sci* 28(4):381-386.

Russell, W. M. S. and R. L. Burch. 1959. *The Principles of Humane Experimental Technique.* London: Methuen.

Saranteas, T., C. Mourouzis, C. Dannis, C. Alexopoulos, E. Lollis, and C. Tesseromatis. 2004. Effect of various stress models on lidocaine pharmacokinetic properties in the mandible after masseter injection. *J Oral Maxil Surg* 62(7):858-862.

Selye, H. 1975. *Stress without Distress.* New York: New American Library.

Silver, L. M. 1995. *Mouse Genetics: Concepts and Applications*. New York: Oxford University Press.

Song, L., W. Che, W. Min-Wei, Y. Murakami, and K. Matsumoto. 2006. Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress. *Pharmacol Biochem Be* 83(2):186-193.

Tournier, J. N., J. Mathieu, Y. Mailfert, E. Multon, C. Drouet, A. Jouan, and E. Drouet. 2001. Chronic restraint stress induces severe disruption of the T-cell specific response to tetanus toxin vaccine. *Immunology* 102(1):515-523.

Ushijima, I., Y. Mizuki, and M. Yamada. 1985. Development of stress-induced gastric lesions involves central adenosine A1-receptor stimulation. *Brain Res* 339(2):351-355.

Willner, P. 1997. Validity, reliability and utility of the chronic mild stress model of depression: A 10-year review and evaluation. *Psychopharmacology* 134(4):319-329.

Würbel, H. 2001. Ideal homes? Housing effects on rodent brain and behavior. *Trends Neurosci* 24(4):207-211.

3

Recognition and Assessment of Stress and Distress

INTRODUCTION

Recognition of distress in laboratory animals requires knowledge of what is normal for the species and strain used. Genetically modified animals should be evaluated in reference to the normality of their genotype.

Most vertebrate species routinely experience some type of distress either in natural settings (e.g., during a predator attack) or as part of normal development (e.g., following natural maternal separation in rhesus monkeys; Berman et al. 1994). The recognition of distress in laboratory animals, however, requires an understanding of what is "normal" for the species being studied. In this chapter we consider the use of behavioral and physiologic variables to recognize stress and distress.

Lab animals should behave according to species-specific normal behavioral, morphologic, and physiologic values (see Novak and Suomi 1988 and Snowdon and Savage 1989 for a discussion of psychological well-being in captive nonhuman primates). Species-specific normative ranges have been established for many parameters (e.g., hematocrit, blood glucose, body temperature, heart rate, blood pressure, respiration rate). Standardized growth curves and weight ranges can be obtained from laboratory animal suppliers for most species.

Recognition of stress and distress in laboratory animals requires an understanding of the species-, gender-, and age-specific norms, because the normal range of some of these variables may vary as a function of gender, age, physiological state, or genetic characteristics. Values outside

normalcy, therefore, may or may not serve as clinical indicators of a disease state. Various transgenic and knockout mice that exhibit severe behavioral and physiological phenotypes appear abnormal relative to their control littermates, but are *normal* for their genotype. For example, it is appropriate to evaluate Huntington's disease transgenic mice for signs of stress and distress only relative to their own "normal" behavior, taking into account their particular genetic makeup, their abnormal motor patterns, and reduced weight gain (Mangiarini et al. 1996).

BEHAVIORAL RECOGNITION OF STRESS AND DISTRESS

Normal Behavior

Many parameters have an effect on species-specific normal behavior and should be taken into consideration when behavioral characteristics are used to determine normalcy or the presence of stress and distress. Animals exhibit a variety of behavioral changes as part of the normal aging process. Males and females differ in the baseline values of many stress markers. Inbred murine strains differ in almost every behavioral, sensory, motor, and physiological trait studied and each inbred strain may respond to stress differently. Similar behavioral differences in response to stress have been observed in primates. Genetically engineered phenotypes need to be considered when assessing stress and distress in transgenic and knockout animals. The maternal environment and rearing experiences of the offspring affect their future responses to stress and distress. Special physiological states, such as impending parturition, are defined by state-specific behaviors. Housing conditions may also modify species-specific behavioral patterns. Behavioral normalcy is further characterized by the absence of bizarre or atypical patterns of species-specific behavior. The presence of stereotypies usually implies suboptimal environments and possibly poor animal welfare.

The identification of species-typical behavior often comes from ethograms developed by researchers to describe the kinds of behavior that animals display in various settings (Bronson 1979; for more references see Additional References). While the use of species-typical behavior as a normative benchmark has considerable value (Latham and Mason 2004), it does have limitations. First, the full range of species-specific behaviors cannot be recreated (or allowed to be expressed) in the laboratory animal care facilities as some types of behavior observed in natural settings (e.g., severe aggression) are clearly undesirable from a laboratory management perspective. Second, species-typical behaviors are neither invariant nor universal, as both the frequency and the presence of such behaviors vary

as a function of age, gender, physiological state, and genetic constitution. Third, rearing practices and housing environments may affect expected typical behavior.

1. Age: Many young mammals engage in high levels of social play whereas adults rarely do. Thus, play may be normal for young animals but not necessarily so for adults (Ruppenthal et al. 1974; Vanderschuren et al. 1997). All animals display physiological, behavioral, and cognitive changes as they age. Some of these changes, for example changes in coat/hair appearance and locomotor ability, are overt and easily recognizable. Many laboratory animals display an age-related decline in exploratory activity, which is sometimes correlated to weight gain (as observed, for example, in mice of various strains; Ingram 2000; Ingram et al. 1981). In addition, a number of neurosensory, cardiovascular, endocrine, gastrointestinal, musculoskeletal, and reproductive changes occur with aging, some of which cannot be directly observed in the living animal. Such age-related changes (e.g., hearing and vision deficits) have been documented for a number of murine strains (Hawkins et al. 1985; for more references see Additional References), while cognitive deficits have been reported in aging mice and rats. It is postulated that some of these changes may be gender- and strain-related (Decker et al. 1988; Fischer et al. 1992; Frick et al. 2000). Changes in pain sensitivity and in emotional behavior that may have direct implications for stress and distress have also been reported in aging animals (Berry et al. 2007; Lamberty and Gower 1992).

2. Gender: Female mammals generally care for infants, whereas the extent of male involvement varies across species. Thus, species-typical behavior may be gender-biased. Moreover, gender-related differences in stress markers can be profound and occur in all vertebrate species. For example, female rats and mice exhibit marked elevations in basal and stress-induced corticosterone release relative to males, although these are buffered by high levels of corticosteroid-binding globulin, thus making free corticosterone levels similar in both sexes (McCormick et al. 1995). Absolute corticosteroid levels in females fluctuate in relation to the stage of estrus, presumably affected by circulating levels of estrogens (Figueiredo et al. 2002). Thus, assessment of HPA activity as a measure of stress (see below) needs to account for the gender of the animal and the type of steroid measurement (i.e., total [plasma] or free [saliva]). Males and females also appear to differ in other aspects of their stress response(s); for example, while females have greater anhedonic and HPA axis responses to chronic mild stress than males, they score lower on tests of behavioral depression caused by chronic stress exposure (Dalla et al. 2005).

3. Genetic traits: Genetic variability among many animal species complicates our understanding of the effects of stress and distress in laboratory animals. Multiple studies in the mouse have shown that generalizations even across a single species can be problematic. Selective breeding has produced hundreds of inbred mouse strains, providing extensive genetic and phenotypic variability (Beck et al. 2000; Silver 1995). A mouse strain is classified as *inbred* after 20 inbreedings (that is, 20 generations of brother x sister or offspring x parent matings), at which point its members are virtually genetically identical because at the 20th or subsequent generations all animals are traceable to a single breeding pair. One cannot assume that mice from different inbred strains are alike (or even similar), perform identically, or experience and react uniformly to stimuli—stressful or otherwise. In fact, inbred strains of mice differ in almost every behavioral, sensory, motor, and physiological trait studied to date, such as anxiety, learning and memory, brain structure and size, visual acuity, acoustic startle, exploratory behavior, alcohol sensitivity, depression, pain sensitivity, and motor coordination (Crawley et al. 1997; for more references see Additional References). What is typical for one strain—for example, high levels of play behavior or social interaction (Moy et al. 2004) or novelty seeking and exploratory behavior (Bolivar et al. 2000; Kliethermes and Crabbe 2006)—may not be characteristic of another.

For these reasons different inbred murine strains respond to stress differently and thus may well experience distress in different ways. Indeed, a number of behavioral studies provide evidence that strain differences in distress susceptibility are likely. For instance, inbred strains differ in performance on anxiety, depression, and fear learning assays (Balogh and Wehner 2003; for more references see Additional References). Correlating behavioral performance across such matrices can provide some indication of basic genetic differences among strains in response to stressful situations (Ducottet and Belzung 2005). When exposed to a month of unpredictable mild stress (e.g., cage tilting, damp bedding, lights on for a short period during the dark phase) most strains groom themselves less resulting in poor fur condition, while only a few display heightened aggression levels (Mineur et al. 2003). In general, inbred strain differences appear in the stress-induced hyperthermia model (Bouwknecht and Paylor 2002; van Bogaert et al. 2006) and in stress-invoked autonomic responses (body temperature and heart rate), although the latter are also a function of the intensity of the insult applied (van Bogaert et al. 2006). Behavioral differences have also been observed in primates. High reactor monkeys[1] are much less likely

[1]It is now well established that there are marked individual differences in reactivity among nonhuman primates when animals are exposed to novel situations or to relatively minor changes in their social or physical environment. Some rhesus monkeys (~20%) respond to

to explore a novel stimulus than low reactors (Suomi 2004). Moreover, because even within genetically diverse species individual animals will vary on many dimensions, high levels of exploration may be the norm for some but not for others.

4. Transgenic and knockout mouse models: Many genetic mouse models have intentional or incidental behavioral and/or physiological phenotypes relevant to stress. Disturbances in genes associated with brain stress-regulatory systems can elicit stress hyposensitivity (e.g., deletion of the corticotrophin-releasing hormone [CRH] gene; Muglia et al. 1995) or stress hypersensitivity (e.g., overexpression of CRH; Stenzel-Poore et al. 1994). Moreover, there are any number of transgenic/knockout phenotypes that affect behavioral or physiological indices of stress without producing overt stress or distress. For example, deletion of the S6 kinase gene produces a remarkably small animal, not because of the animal's "failure to thrive" but rather because absence of this powerful cell-size regulator results in a smaller size of otherwise healthy cells (Thomas 2002). Thus, expressed phenotypes need to be considered when assessing stress and/or distress in genetically engineered animal models because their presence may be even more difficult to recognize and diagnose in these animals than in their control littermates.

5. Rearing and postnatal separation: In most mammals, the early environment of the young animal is defined by the presence of its mother; therefore maternal characteristics can have a profound impact on the future behavior of adult offspring. There is ample scientific evidence that maternal environment can be an important epigenetic determinant of physiology and behavior, and should be considered as a variable for assessment of stress and distress. Offspring are generally reared with their mothers and may also be reared in larger social groups that include other offspring as well as adult males and females. Some species- or strain-typical behaviors, such as cross fostering, in which the offspring of one species are reared by the parents of another species or of the same species but a different strain, are more susceptible to parent-related environmental manipulations. The extent to which cross fostering may produce distress in the offspring

---

relatively mild environmental stressors with unusual behavioral disruption and physiological arousal including prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, as assessed by plasma cortisol and adrenocorticotropic hormone (ACTH), increased cerebrospinal fluid levels of the norepinephrine metabolite 3-methoxy-4-hydroxyphenylglycol, heightened sympathetic nervous system activity as reflected in altered heart rate rhythms, and abnormal immune system response (Coe et al. 1989; Higley et al. 1991). The same stressors elicit only minor behavioral reactions and transient physiological responses in the remainder of the population (Suomi 2004).

depends on the degree to which parental care varies across the two species (or strains) in question. In birds, cross fostering can be relatively benign (e.g., rearing of green finches' offspring by canaries; Guettinger 1979). In other cases, however, cross fostering may complicate the assessment of stress and distress, as cross-fostered rats, mice, and goats frequently exhibit behaviors more similar to the adoptive mother strains (Ahmadiyeh et al. 2004; Anisman et al. 1998; Kendrick et al. 2001). In rats, female offspring of dams bred for high licking and grooming that have been reared with their biological mothers will themselves provide extensive maternal care of their own pups. In contrast, if female offspring of high licking and grooming dams are instead cross fostered with low licking and grooming (i.e., "poor") mothers, they will subsequently provide little maternal care to their own offspring, resulting in behavioral and physiological changes that persist into adulthood (Francis et al. 1999). Recent research into the effects of maternal behavior on such developmental traits as DNA methylation, an epigenetic mechanism that alters gene expression, has shown that maternal environmental programming (for example, high or low grooming) affects the glucocorticoid receptor gene and possibly the responses of the offspring to stress. Offspring of high grooming mothers (or those cross fostered to them) appeared less responsive to stressful stimuli and had increased expression of these receptors in the hippocampus compared to those raised by low grooming dams (Fish et al. 2004; Weaver et al. 2004). Microarray analysis has shown that more than 900 genes of the hippocampal transcriptome are stably regulated by maternal care (Weaver et al. 2006).

In species such as primates, however, infants may be nursery-reared because of the infant's illness, the mother's failure to care for the infant, or demands of the experimental protocol. The two most common nursery rearing procedures for macaques are peer rearing (i.e., rearing infants together 24 hours a day) and surrogate peer rearing (i.e., rearing infants on inanimate surrogate mothers 24 hours a day with 1-2 hours of daily peer exposure in a playroom setting). Both conditions commence shortly after an animal's birth before a strong attachment has been formed to the mother, and thus infants show little in the way of separation anxiety.

From a developmental perspective, peer rearing appears to confer the greater risk for distress and social maladjustment. Peer-reared monkeys typically show higher levels of mutual clinging and greater fear responses than surrogate-peer-reared monkeys early in life and have difficulty adapting to larger social groups as juveniles (Ruppenthal et al. 1991). Peer rearing has also been associated with impaired immune responses (Coe et al. 1989; Lubach et al. 1995) and, when combined with repeated separations, appears to promote heightened aggressiveness, impaired impulse control, alcohol abuse, and low levels of 5-hydroxyindoleacetic acid (a serotonin metabolite) in cerebrospinal fluid (Ichise et al. 2006). Although

less studied, surrogate-peer-reared monkeys appear to behave more like normally reared monkeys. Indeed, a large comparison study of surrogate-peer-reared monkeys (n=506) to normally reared monkeys (n=1,187) failed to detect any differences in growth, health, survival, reproduction, and maternal abilities between the two groups (Sackett et al. 2002). But because some individuals reared in either condition may be more vulnerable to the development of abnormal behavior than normally reared monkeys, careful observation and ongoing assessments would help guide colony management decisions regarding group composition and enrichment strategies.

A different kind of early rearing experience involves separation from the mother or other attachment figure (e.g., other peers) after a strong attachment has been formed. Such separations may occur both for research purposes or to facilitate weaning. Depending on such variables as the timing of the separation, the nature of the separation environment, and the primate species, separation can induce high levels of stress in infants expressed by vocalizations and heightened activity (Bayart et al. 1990; Jordan et al. 1985; Laudenslager et al. 1990; Levine et al. 1993). It can also alter HPA activity (Levine 2005; Levine and Mody 2003; Parker et al. 2006; Vogt et al. 1980) and immune responses (Laudenslager et al. 1982). Reactions are often stronger when the infant is separated both from its mother and the environment in which it was raised compared to when only the mother is removed from the infant, but this effect can vary by species (Laudenslager et al. 1990). These signs generally disappear when infants are reunited with their mothers or their attachment figures, although neuroendocrine responses may be altered.

6. Physiological state: Many species (such as dogs, sows, rabbits, and mice) need to build nests just before parturition, whereas others do not engage in such behavior (Arey 1997; Broida and Svare 1982; Crawley 2000; Kunkele 1992).

7. Housing: Environmental conditions can modify species-specific behavioral patterns. Adults housed in same sex groups cannot show some aspects of the species-typical physiological repertoire (e.g., mating or parental behavior). Housing conditions (such as cage types and environmental enrichment) affect the amount of time that mice spend engaging in distinct behavioral patterns, as reported by Olsson and Sherwin who, using videorecording, showed that mice in furnished cages (i.e., cages with nesting material, running wheels, nest box, and chew box) "spent less time resting, bar-chewing and bar-circling and more time on exploratory/locomotor behaviors" (Olsson and Sherwin 2006, p. 392). Lack of environmental stimulation or social deprivation adversely impacts normal brain function in rats, such as attenuation of the prepulse inhibition (PPI) behavior elicited by

startling events, and is accompanied by underlying neurochemical changes such as enhanced dopamine activity (Würbel 2001). Based on significantly fewer instances of abnormal behaviors (i.e., stereotypies) encountered in wild-caught animals vs. their captive-bred controls, the argument for a neuroprotective effect of early environmental enrichment against future abnormal behaviors has been made (Lewis et al. 2006). Moreover, studies have shown that dendritic anatomy in young rats was altered in response to a brief 4-day exposure to a complex environment (Wallace et al. 1992) and so was the hippocampus of adult mice in comparison to controls (Kempermann et al. 2002; for more discussion on enrichment see Chapter 4).

Behavioral normalcy is further characterized by the absence of bizarre or atypical patterns of species-specific behavior. Examples of abnormal behavior include excessive barbering observed in mice (Garner et al. 2004; Morton 2002), regurgitation/rumination and coprophagy seen in apes (Nash et al. 1999), or more serious self-injurious behaviors exhibited by rhesus monkeys (Novak 2003). Sometimes, such behaviors represent normal social patterns. For example, coprophagy associated with mother rearing occurs not only in laboratory-housed apes (Nash et al. 1999) but also in the wild where it is postulated to contribute to the reclaiming of unused resources from the feces (Krief et al. 2004). In other cases, however, such patterns are a sign of well-defined diseases or disorders, as, for example, excessive tremors observed in transgenic mice with Huntington's disease (Mangiarini et al. 1996). In yet other instances, abnormal behavioral patterns, such as stereotypies, may result from suboptimal housing environments (Bayne et al. 1992, 2002; Hubrecht et al. 1992; Mason 1991).

Stereotypic behavior is characterized by highly repetitive and ritualistic actions, the function of which is largely unknown. Environments that elicit or enhance stereotypies not as part of defined pathophysiology or disease models are typically suboptimal (Berkson and Mason 1964; for more references see Additional References). Stereotypies vary across species and appear at different times of day and under different conditions (Mason and Mendl 1997). Classic whole-body stereotypies include circling, pacing (dogs, primates), wall bouncing (dogs), and somersaulting and bar chewing (rodents), whereas self- or other-animal-directed stereotypies often involve the limbs or face and include such patterns as digit sucking, paw licking, and overgrooming (Bayne and Novak 1998; for more references see Additional References).

Although there is yet inadequate research on the relationship between distress and stereotypy, a recent meta-analysis of studies linking stereotypy to animal welfare suggests that some stereotypies may function to regulate arousal and possibly reduce distress as "do-it-yourself enrichment" strategies to alleviate the effect of a suboptimal environment (see Mason and

Latham 2004). Overall, however, the presence of stereotypies should be a cause for concern because animals that exhibit such behavioral patterns may not only have experienced some stress or distress in the past but also live in environments that promote or sustain these abnormal behaviors. Moreover, as a study by Krohn and colleagues has shown, stereotypies are probably underreported as they may occur during the night when staff are not present, or cease when staff enter a room (Krohn et al. 1999). If, in fact, the presence of stereotypies is being investigated, then more sophisticated methods such as closed-circuit television or videorecording, or simpler diagnostics such as partially reversed light cycles, would enable staff to observe nocturnal animals during their most active periods in order to document instances of abnormal behavior (Hubrecht 1997).

Abnormal Behavior and Clinical Signs

Recognition of distress should be derived from intimate knowledge of the species' or strain's normal behavior and may be based on (1) clinical signs and/or (2) significant deviation from the expected behavioral repertoire. Some clinical signs (e.g., changes in temperature, respiration, feeding behavior) indicate an abrupt onset of distress while others (e.g., weight loss) develop over a longer period of time and may serve as warnings. A thorough clinical examination with references to baseline effects of age, gender, genotype, etc., is necessary to establish the presence of distress, while an abrupt and marked change in behavior lasting more than a few days may also indicate a disease state. While the presence of stereotypies is undesirable, the relationship between stereotypic behavior and distress remains largely unknown. Preventing the development of stereotyped behavior by providing species-specific appropriate environments is likely to result in improved welfare.

Assuming that an animal's behavior has been well characterized, indications of distress may include certain clinical signs or marked change from the individual animal's usual behavioral repertoire (Morton and Griffiths 1985; see score sheet examples in Appendix). An abrupt and marked change in behavior lasting more than a few days may also indicate the presence of a disease state in addition to distress, particularly if these changes occur in conjunction with severe reductions in normal daily activities such as feeding behavior, sexual behavior, maternal behavior, or attention to threat. Conversely, animals may exhibit increased activity associated with unusual motions (e.g., head rubbing) or unusually high levels of certain behaviors (e.g., scratching). Even marked changes in behavior, however, must be evaluated in context. For example, females usually exhibit decreased activity the first day following parturition, an expected behavior.

Clinical Signs of Distress

Clinical examination to establish the presence of distress should focus on, but *not be limited to*, the following: signs of abnormal respiration (shallow, labored, or rapid); assessment of grooming and hair coat (piloerected or greasy, possibly reflecting reduced grooming); examination of the eyes (runny, glassy, or unfocused); examination of motor postures (hunching or cowering in the corner of the cage, lying on one's side, lack of movement with loss of muscle tone); absence of alertness or quiescence (inattention to ongoing stimuli); changes in body weight; the ability or failure to produce urine or feces; unusual features of urine (volume, smell, and color) or feces (quantity, consistency, and color); the presence of vomit; the status of the animal's appetite and water intake; and intense or frequent vocalizations (Bennett et al. 1998; Fortman et al. 2002; Fox et al. 2002). It is appropriate to evaluate some of these signs in context, as, for example, rapid breathing could result from vigorous activities such as playing or running on the wheel, lying down may occur as part of social grooming (e.g., among macaques), weight loss is often associated with advanced age, and some mammals raise their hair (piloerection) while eating. In addition, clinical evaluation and diagnosis should consider species, age, gender, physiological state, and genetic variables (Bennett et al. 1998).

While some of the clinical signs described above (e.g., respiratory changes, changes in fecal material and/or in urine) are more relevant to the acute onset of a distressful state, other measures may serve as potential early warning signs of distress (e.g., rapid body weight changes in the absence of dietary modifications). Significant and unexpected changes in weight in either direction may be indicators of altered endocrinological, immunological, or neurological parameters. Indeed, the relatively sudden loss of 25% body weight of a nonhuman primate is one of the parameters used to determine humane endpoints in primate research (Association of Primate Veterinarians 2008).

This view should not be applied to caloric restriction research protocols where animals may be subject to controlled diets that reduce their weight by as much as 15-20% (Heiderstadt et al. 2000). Such protocols are widely used in gerontology research where diet has been shown to slow aging, extend lifespan, and reduce the incidence of age-related diseases in rodents (Goto et al. 2002; for more references see Additional References), while beneficial effects have also been observed in nonhuman primates (Ingram et al. 2007). Moreover, sensory-motor function and learning studies may use caloric or water restriction as a motivational tool (Heiderstadt et al. 2000; Smith and Metz 2005). In these studies regular monitoring of body weight is essential to ensure that animals either do not fall below an accepted weight

range or, in the case of young animals, gain the appropriate body weight for their age.

Behavioral Signs of Distress

It has been suggested that abnormal behavior, such as stereotypies, is a marker for distress (Dawkins 1990). It remains unclear at this time whether any or all abnormal behaviors qualify as indicators of distress. Several alternative (and largely speculative) hypotheses attempt to explain the occurrence of stereotypic behavior in animals (Mason and Latham 2004; Tiefenbacher et al. 2005). Among these, the stimulation hypothesis suggests that when sensory motor input is low, possibly due to existing (i.e., nonstimulating, poor) housing arrangements, animals engage in stereotypic behavior to self-provide increased sensory-motor input (Sherwin 1998). For example, when cage size constrains normal movements, some animals may respond by developing stereotyped pacing in order to satisfy their need for activity (Draper and Bernstein 1963). The habit hypothesis suggests that although stereotypic behavior may have originally arisen in response to stress or distress, it persists as a habit uncoupled from the situation that originally produced it (Dantzer 1986; Mason 1991). Those who favor the arousal reduction hypothesis suggest that stereotypic behavior may serve to calm the animal and thereby avoid distress (reviewed in Mason 1991). Research shows that in some humans and nonhuman primates, even more serious forms of abnormal and self-injurious behavior may function to reduce arousal (Tiefenbacher et al. 2005). The arousal reduction hypothesis is consistent with the view that while an underlying stress or distress state may have initially caused abnormal behavior, eliminating the behavior may be neither desirable nor possible because the stereotypy may sometimes prevent the onset of distress.

Preventing the development of stereotyped behavior by providing the animals with species-specific appropriate environments is obviously desirable and likely to result in improved welfare, especially as enrichment "therapy" may reduce but will not cure the abnormal behavior (van Praag et al. 2000; Wolfer et al. 2004). Although recent studies suggest that stereotypical animals may experience psychological distress due to a putative common mechanism between stereotypy, schizophrenia, and autism, the relationship between stereotypic behavior and distress remains largely unknown and is in need of further study (Garner 2006; Garner and Mason 2002; Garner et al. 2003; Mason 2006).

Behavioral Signs of Stress

As mentioned in Chapter 2, stress is ubiquitous, it can occur in both pleasurable and aversive situations, and its physiological parameters are well established. Our knowledge of the behavioral correlates of stress, however, is considerably smaller. The behavioral changes observed in a stressed animal (as opposed to a distressed one) may be more subtle and variable, depending on the environmental conditions in which the behavior is being evaluated. In addition to recognizing an animal's normal patterns of behavior, the observer must be well trained and knowledgeable about the normal species-specific behavior in the context of species, strain, gender, and physiological state. Types of behavior commonly explored to investigate the presence of stress include open-field activity, movements in an elevated plus maze, changes in innate behaviors (e.g., movement, grooming, feeding, sexual behavior), defensive behaviors (to external threats), and avoidance/escape (Beck and Luine 2002; for more references see Additional References).

PHYSIOLOGIC MEASURES OF STRESS AND DISTRESS

Endocrinological Parameters

One of the primary endocrinological systems involved in the stress response is the hypothalamic-pituitary-adrenal (HPA) axis, which reacts to stress by releasing glucocorticoids. Glucocorticoid levels can be used as indicators for the impact and strength of a stressor, with two caveats: (1) they cannot inform as to the type of stressor (positive or aversive) that stimulates the HPA and (2) most sampling procedures are themselves stressful to the animals, thereby confounding the measurements. Therefore, the assessment of distress based on glucocorticoid levels has limitations, especially under the unproven assumption that a certain glucocorticoid concentration indicates the presence of distress. Furthermore, stress or distress may exist without the concomitant activation of the HPA axis.

Glucocorticosteroids

The hypothalamic-pituitary-adrenal (HPA) axis, often referred to as the "stress response system", plays an important role in an organism's reaction to stressors. In response to a stressful situation the hypothalamic paraventricular nucleus synthesizes corticotrophin-releasing hormone (CRH), which is released into the median eminence and travels to the anterior pituitary where it causes the release of adrenocorticotropic hormone (ACTH) into the circulatory system. ACTH then acts selectively on specific

receptors in the adrenal cortex, resulting in the release of glucocorticoids (cortisol or corticosterone), which mobilize energy stores in response to the perceived stress. When the stressor is removed or otherwise adapted to, glucocorticoids bound to receptors in the hypothalamus and pituitary initiate negative feedback that causes a decrease in the production and release of CRH and ACTH, thus terminating the hormonal response and completing the negative feedback loop (Meaney et al. 1996; Miller and O'Callaghan 2002). It should be noted that the HPA axis affects many hormonal and neural systems and plays a role in modulating the immune system.

Both positive and negative stimuli activate the HPA axis with short- and long-lasting effects. For example, exposure to novel stimuli may elicit exploratory behavior and brief activation of the HPA axis. In contrast, prolonged or repeated stressors, such as social separation of young from their mother, generally elicit strong protest reactions and activation of the HPA axis (Levine 2005; Levine and Mody 2003; Vogt et al. 1980). In the first instance, homeostasis is quickly restored, whereas in the latter case animals may be subjected to chronic HPA changes associated with neuroendocrine stress resistance that persists even after animals are returned to their mother (Parker et al. 2006).

Glucocorticoid levels (usually corticosterone in rodents, cortisol in other species) are used as indicators of the strength and impact of a stressor. Meaningful interpretation of these values, however, presents significant challenges. Glucocorticoids are typically measured in blood serum or plasma but can also be quantified in saliva, urine, feces, and hair (Abelson et al. 2005; for more references see Additional References).

Blood sampling requires venipuncture and possibly other stressful procedures such as handling, transport, capture, restraint, needle stick(s), and sedation. Unless animals are habituated to blood sampling, the method itself can activate the HPA axis thereby confounding assay results. A less stressful sampling method involves measuring glucocorticoid levels in hair. Hair samples are obtained by shaving hair from a particular region (usually the nape of the neck) and then shaving the hair once again after a defined period of regrowth (for a discussion of the method in primates see Davenport et al. 2006). Although this procedure requires the animals' habituation, restraint, or sedation, unlike venipuncture the stress caused by hair collection does not confound the measurement. Similarly, saliva collection may impact animals less if they have been habituated to the process (Lutz et al. 2000). Urine and feces are collected after excretion from the body and so probably have the least impact, unless animals are not habituated to the special metabolic cages used for sample collection.

The type of sample obtained and the time frame it reflects may also influence results. Blood and saliva yield an index of stress at one brief moment in time (point samples) and are, therefore, influenced by circadian

variation (Windle et al. 1998b), making it crucial that samples be collected at the same time each day (e.g., at the nadir of the rhythm). In contrast, urine and feces yield an index of stress reactivity over hours or possibly several days (steady-state samples) and are, therefore, less vulnerable to circadian variation. To date, only hair can provide a chronic index of stress covering a period of several months or more. Assessment of cortisol in hair is presumably unaffected by circadian variation and can be obtained at any time of day.

The above information is relevant for understanding and interpreting what might be revealed about stress and distress by examining the activation of the HPA axis. The two most likely ways to assess distress are to (1) examine differences in basal glucocorticoid levels and identify animals outside a "normal range" or (2) obtain glucocorticoid levels before and after the imposition of a stressor. The first approach is problematic because it assumes that a certain concentration of glucocorticoids indicates distress, although there is no scientific evidence to support this assumption. Moreover, as many sampling methods may themselves activate the stress response, there are no standardized ranges for basal glucocorticoid concentrations. The second approach is also problematic for two reasons: first, the putative relationship between the magnitude of change in glucocorticoid concentrations and distress has not been established; and second, both positive and aversive stimuli activate the HPA axis. Finally, the development of stress or distress is not necessarily associated with activation of the HPA axis, as hormonal changes are not necessarily present under all clearly stressful conditions. For example, animals that experience chronic neuropathic pain do not exhibit changes in circadian corticosteroid levels or oscillations in HPA responsivity to restraint, despite the presence of neuropathic pain markers (mechanical allodynia and hyperalgesia) and activation of central pain and stress circuits in the amygdala (Bomholt et al. 2005; Ulrich-Lai et al. 2006).

Other "Stress" Hormones

As is the case with the hormones of the HPA axis, stressors alter the secretion of other endocrine factors (e.g., prolactin, growth hormone, luteinizing hormone, α-melanocyte stimulating hormone [α-MSH], and oxytocin). Serum levels of these hormones can be effectively used to monitor the temporal dynamics of stress responses. While some (prolactin, α-MSH, oxytocin) increase during stress, others decrease (growth hormone, luteinizing hormone, prolactin), depending on the animal species and the physiological state in which stress occurs (Armario et al. 1984; for more references see Additional References). Due to the fact that these hormones are also released in response to other stimuli (e.g., suckling of young, ultra-

dian or circadian variations), it is necessary to take into consideration and control for their normal patterns of secretion in order to accurately interpret their concentration levels. Moreover, their usefulness is subjected to the same limitations as discussed above, although chronic indwelling vascular catheters and automated blood collection systems may circumvent this limitation to some degree (Abelson et al. 2005; for more references see Additional References).

Neurological Parameters

Stressors activate the autonomic nervous system, specific brain areas, and various neurotransmitters, yet a cause and effect relationship has not yet been firmly established. Candidates for the role of a master integrator include the region of the amydgala and the neuropeptide corticotrophin-releasing factor.

The Autonomic Nervous System

Many different types of stressors cause the rapid activation of the sympathetic division of the autonomic nervous system (ANS) (Blanc et al. 1991; for more references see Additional References). This activation leads to increased cardiac output via increased heart rate and stroke volume; redistribution of blood flow from splanchnic, renal, and cutaneous vascular beds to active muscle; increased mobilization of nutrients; and increased heat production. Some stressors may also increase the activity of the parasympathetic division, affecting both core body temperature and the gastrointestinal system (e.g., disturbed intestinal absorption, gastric ulceration, colitis; Johnson et al. 2002; for more references see Additional References).

Direct monitoring of autonomic activity to assess the presence of distress in conscious animals is technically challenging, while indirect measures are somewhat easier to acquire (Li et al. 1997; Randall et al. 1994; Zhang and Thoren 1998). For example, telemetry in conscious, unrestrained animals is an effective method for the continuous monitoring of physiologic alterations in heart rate, respiration, blood pressure, ECG, and body temperature (Akutsu et al. 2002; for more references see Additional References). Once again, however, changes in these parameters do not necessarily indicate stress as they may result from nonstressful stimuli (e.g., circadian variations).

Neurotransmitters

Considerable effort has been directed at exploring the neurotransmitter systems and brain areas activated in response to stress as different insults

activate separate but specific patterns of transmitters, modulators, and brain locations. An active area of investigation has been the identification of a basic core neural circuit that is activated by all stressors. A common approach has been to divide stressors into categories, hypothesizing that each category would activate a particular set of neural structures. The stressors are classified as either "processive" (i.e., stressors that require interpretation by higher brain structures) or "systemic" (i.e., stressors evoked by an immediate physiological threat; Herman and Cullinan 1997). However, the neural response has been heterogeneous for both these two as well as more narrowly defined categories. For example, Serrats and Sawchenko administered either lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB) to rats in order to study brain activation patterns using *c-fos* induction as a measure of neural activity. Although both LPS and SEB activated some of the same brain structures, they produced distinctly different patterns of neural activation (Serrats and Sawchenko 2006).

Research indicates that there are few, if any, transmitters and modulators that are *not* activated in some region of the brain by some stressor. Monoaminergic circuits, such as noradrenergic neurons, projecting from the locus coeruleus (Aston-Jones et al. 1996), serotonergic neurons projecting from the dorsal raphe nucleus (Lowry 2002), and dopaminergic neurons projecting from the ventral tegmental area (Pezze and Feldon 2004) are particularly stress-responsive. Research on neuropeptides has focused on CRH (Nemeroff and Vale 2005), endogenous opioids (Ribeiro et al. 2005), and neuropeptide Y (Heilig 2004). CRH may be the key central integrator of the stress response as CRH-containing neurons in the paraventricular nucleus of the hypothalamus are the primary common path in the neural regulation of both the HPA and autonomic responses to stressors. In addition, CRH is found in the central amygdaloid nucleus, an important node in regulating behavioral alterations in response to fear (see below for additional information on the fear response).

A somewhat different experimental approach to elucidate the interaction between stressors, neurotransmitters, the brain, and behavioral patterns has been to identify the neural circuit that mediates a specific behavior. For example, the neural circuitry involved in fear conditioning is well known. When a neutral stimulus, such as a light or a tone, is followed closely by an aversive stimulus such as a foot shock, it elicits a fear response. The association between the neutral and aversive stimuli is formed in the basal and lateral nuclei of the amygdala via an N-methyl D-aspartate (NMDA)-dependent long-term potentiation (LTP)-like process. The information is subsequently transmitted via the central nucleus of the amygdala to the proximate mediators of the particular behavioral and physiological constituents of fear (Davis and Whalen 2001) as, for example, to hypothalamic nuclei that regulate respiration. However, many of the fear-modulating

factors alter the activity in structures that project to the top of the central nucleus of the amygdala (Sotres-Bayon et al. 2006), underscoring the amygdala as the key integrative site for fear.

In contrast, much remains to be learned about the neural control of defense responses to threat. For example, depending on circumstances, external threats such as the presence of a predator may result in flight, freezing, or other defensive behaviors. While the involvement of the midbrain periaqueductal gray is well known (Keay and Bandler 2002), inactivation of the brain areas typically responding to a threat from a predator reduces the defense response(s) elicited by predator odor or exposure (Blanchard et al. 2005; Canteras 2002), which may differentially impact predator defense and shock stimuli responses.

Immunological Parameters

The relationship between stress, distress, and the immune system is very complex. Acute stress usually activates innate immune responses (i.e., nonspecific immunity), but it may either increase or inhibit adaptive immunity. On the other hand, chronic stressors suppress adaptive immune responses. Activation of various types of immunity-related cells may be used as an indicator of immune system-stress interaction.

Signaling pathways link the brain with the immune system thereby allowing stress and distress to influence immune function. Immune system cells such as lymphocytes and macrophages express receptors for a variety of hormones and neurotransmitters, while the spleen and thymus are innervated by the autonomic nervous system (Felten et al. 1985; Sanders et al. 2001). The complex nature of these influences, however, does not permit simple generalizations such as "stress/distress suppresses immune function". The immune responses elicited depend on the type, duration, and intensity of the stressor; the species, strain, age, and gender of the animal(s); and the aspect of immunity examined.

Acute Stress/Distress and Immunity

The principles that determine whether acute stressors inhibit or potentiate adaptive immunity are currently unknown. Nevertheless, adaptive immune responses that involve antigen recognition by T cells are invariably affected in acute stress. As Fleshner and colleagues have shown, rats stressed by inescapable tail shock failed to expand a subset of T cells and produced reduced quantities of IgM and IgG antibodies (Fleshner et al. 1995). In contrast, restraint at the time of immunization was shown to facili-

tate immunological memory due to elevated counts of memory and effector helper T cells (Dhabar and Viswanathan 2005).

In contrast, the innate immune response is most often enhanced in response to acute stress, including during stress conditions identical to those that interfere with specific immunity (Deak et al. 1999; Fleshner et al. 1998). A variety of stressors have been reported to increase macrophage function and elevate levels of known pro-inflammatory mediators such as interleukin-1 and tumor necrosis factor (O'Connor et al. 2003). Acute phase protein levels are similarly elevated as these cytokines also initiate the acute phase response. In addition, acute stressors potentiate or even directly elicit the sickness response, a set of behavioral and physiological changes (including fever, increased sleep, reduced social interaction and physical activity) that occur during infection (Dantzer 2004; Maier and Watkins 1998).

Chronic Stress/Distress and Immunity

Chronic or repeated stress has been shown to suppress adaptive immunity (Tournier et al. 2001), but not much is known of its effects on innate immunity. Studies looking at the effects of stress on disease outcome rather than on immune responses have shown that stress can either increase or decrease disease severity depending on conditions and variables measured. Disease progression can be either inhibited or facilitated depending on the precise occurrence of the insult, as the timing of stressor exposure relative to disease onset is often critical (Johnson et al. 2006).

In addition to assays that measure T-cell proliferation, natural killer cell cytotoxicity, or B-cell activation and antibody production as indicators of adaptive immunity-stress interaction, one can also measure the capacity of polymorphonuclear cells to produce a respiratory burst in vitro. Research has shown that the functional capacity of leukocytes from stressed animals is suppressed, thus diminishing their "coping capacity" (as defined by the production of oxygen free radicals; McLaren et al. 2003).

ASSESSMENT OF DISTRESS

Clinical signs interpreted through relevant animal behavior and physiological states are the most reliable distress measures. Distress evaluation is crucial when research animals are purposefully exposed to stressful conditions or when animals appear distressed unexpectedly. The assessment and subsequent interventions should involve researchers, veterinarians, and technicians and the team should continue its collaboration to develop an intervention strategy once the assessment is completed.

Assessment of distress varies in relation to the species, husbandry conditions, and experimental protocol employed as well as with each individual animal, and is most effectively achieved by the collection of multiple behavioral and physiological parameters and the use of a team approach that includes researchers, veterinarians, and animal caretakers/technicians. While the most reliable distress measures are the clinical signs previously described, identification and interpretation of these results depends on a solid foundation of knowledge of animal behavior and may likely require special training of relevant personnel.

Distress evaluation becomes crucial in two contexts: (1) when the research protocol calls for the animals' exposure to stressful situations known to produce distress; and (2) when any animal unexpectedly shows signs of distress. In the first case, the experimental protocol approved by the Institutional Animal Care and Use Committee (IACUC) generally includes procedures and decision algorithms for distress management. The appropriate intervention will be informed by the stressor's duration and intensity as well as some of the animal's individual characteristics (e.g., species, age, gender). In the second case, additional assessments and monitoring may be necessary.

Once an animal has shown initial signs of distress, there should be immediate communication between the primary investigator, the veterinarian, and the animal care staff to determine whether the distress is related to the study (whether anticipated or unanticipated) or further investigation into its cause is required. The discussion should also include potential interventions (see Chapter 4) and their effects on the objectives of the research project, as they may introduce unknown variables into the study. Options may include removal of the animal from the study population or euthanasia, depending on the severity and prognosis of the distress insult. It is essential to maintain a collaborative relationship and dialogue between those responsible for the care and welfare of the animal throughout the assessment.

The next step is to identify the etiology or trigger of the distress episode by performing a thorough examination of the animal and its environment. The investigation should begin by obtaining information regarding the animal's species, strain, age, gender, and reproductive status. An effective examination should account for species-related differences among natural behaviors, learning abilities, and levels of intelligence, in addition to the ways animals use their senses and communicate. Some species, strains, or breeds are predisposed to certain behavioral problems or have certain behavioral phenotypes, or an individual animal's characteristics may affect both the development and alleviation of its distress.

Physical examination and appropriate diagnostic tests for all distressed animals can help determine whether an underlying medical condition is the primary cause of distress. A review of the medical and investigator records

is an important part of the process, as background information and history may enable the veterinarian to determine whether preexisting medical conditions were resolved. An examination of colony records and interviews with animal care staff may help pinpoint possible environmental triggers. Other causes for consideration include husbandry and handling procedures, the behavior of other animals in the room, temperature variances, noises, vibrations, and odors, as well as any specific research-related (i.e., protocol specifications) or investigator-related (i.e., disturbance of housing routine) activities.

Clinical signs should initially be examined in a relatively undisturbed animal in order to assess the animal's natural unprovoked behavior (e.g., appearance, behavior, posture, respiratory rate and pattern). The animal showing signs of distress is then observed more closely followed by gentle handling and examination to measure body weight, body condition and temperature, heart rate, dehydration, and alertness. For some parameters, the degree of change from the normal scale is a useful evaluation indicator, the assumption being that the greater the deviation from normalcy, the greater the impact. For example, an animal may lose 5, 10, 20, or even 40 percent of its body weight, or its temperature may rise (or fall) by several degrees above (or below) normal. Clinical assessments can also be supplemented by video records of the animal in the colony room or laboratory testing environment.

A team approach during assessment is crucial. The assessment of distress and subsequent interventions should involve researchers, veterinarians, and technicians, as they are often the first to observe signs of distress in individual animals. The team should similarly collaborate to develop an intervention strategy once the assessment is completed.

REFERENCES

Abelson, K. S., B. Adem, F. Royo, H. E. Carlsson, and J. Hau. 2005. High plasma corticosterone levels persist during frequent automatic blood sampling in rats. *In Vivo* 19:815-819.

Ahmadiyeh, N., J. L. Sklone-Wilcoxon, J. S. Takahashi, and E. E. Redei. 2004. Maternal behavior modulates X-linked inheritance of behavioral coping in the defensive burying test. *Biol Psychiat* 55:1069-1074.

Akutsu, H., T. Kikusui, Y. Takeuchi, K. Sano, A. Hatanaka, and Y. Mori. 2002. Alleviating effects of plant-derived fragrances on stress-induced hyperthermia in rats. *Physiol Behav* 75:355-360.

Anisman, H., M. D. Zaharia, M. J. Meaney, and Z. Merali. 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int J Dev Neurosci* 16:149-164.

Arey, D. S. 1997. Behavioral observations of peri-parturient sows and the development of alternative farrowing accommodation: A review. *Anim Welf* 6(3):217-229.

Armario, A., J. M. Castellanos, and J. Balasch. 1984. Dissociation between corticosterone and growth hormone adaptation to chronic stress in the rat. *Horm Metab Res* 16:142-145.

Association of Primate Veterinarians. 2008. APV position statement on humane endpoints of nonhuman primates in research.

Aston-Jones G., J. Rajkowski, P. Kubiak, R. J. Valentino, and M. T. Shipley. 1996. Role of the locus coeruleus in emotional activation. *Prog Brain Res* 107:379-402.

Balogh, S. A. and J. M. Wehner. 2003. Inbred mouse strain differences in the establishment of long-term fear memory. *Behav Brain Res* 140:97-106.

Bayart, F., K. T. Hayashi, K. F. Faull, J. D. Barchas, and S. Levine. 1990. Influence of maternal proximity on behavioral and physiological responses to separation in infant rhesus monkeys *(Macaca mulatta)*. *Behav Neurosci* 104(1):98-107.

Bayne, K. A. L. and M. A. Novak. 1998. Psychological disorders. In *Nonhuman Primates in Biomedical Research*, B. T. Bennett, C. R. Abee, and R. Henrickson, eds. Vol. II: Diseases. New York: Academic Press. 485-500 pp.

Bayne, K. A. L., S. Dexter, and S. Suomi. 1992. A preliminary survey of the incidence of abnormal behavior in rhesus monkeys *(Macaca mulatta)* relative to housing condition. *Lab Anim* 22(5):38-44.

Bayne, K. A. L., J. A. Mench, B. V. Beaver, and D. B. Morton. 2002. Laboratory Animal Behavior. In *Laboratory Animal Science*, ACLAM series, 2nd ed. New York: Academic Press. 1240-1264 pp.

Beck, K. D. and V. N. Luine. 2002. Sex differences in behavioral and neurochemical profiles after chronic stress: Role of housing conditions. *Physiol Behav* 75(5):661-673.

Beck, J. A., S. Lloyd, M. Hafezparast, M. Lennon-Pierce, J. T. Eppig, M. F. W. Festing, and E. M. C. Fisher. 2000. Genealogies of mouse inbred strains. *Nat Genet* 24:23-25.

Bennett, B., C. Abee, and R. Henrickson, eds. 1998. *Nonhuman Primates in Biomedical Research: Diseases*. New York: Academic Press.

Berkson, G. and W. A. Mason. 1964. Stereotyped behaviors of chimpanzees: Relation to general arousal and other activities. *Percept Motor Skill* 19:635-652.

Berman C. M., K. L. R. Rasmussen, and S. J. Suomi. 1994. Responses of free-ranging rhesus monkeys to a natural form of social separation. I. Parallels with mother-infant separation in captivity. *Child Dev* 65(4):1028-1041.

Berry, A., F. Capone, M. Giorgio, P. G. Pelicci, E. R. de Kloet, E. Alleva, L. Minghetti, and F. Cirulli. 2007 Deletion of the life span determinant p66 (Shc) prevents age-dependent increases in emotionality and pain sensitivity in mice. *Exp Gerontol* 42(1-2):37-45.

Blanc, J., M. L. Grichois, and J. L. Elghozi. 1991. Effects of clonidine on blood pressure and heart rate responses to an emotional stress in the rat: A spectral study. *Clin Exp Pharmacol P* 18(10):711-717.

Blanchard, D. C., N. S. Canteras, C. M. Markham, N. S. Pentkowski, and R. J. Blanchard. 2005. Lesions of structures showing FOS expression to cat presentation: Effects on responsivity to a cat, cat odor, and nonpredator threat. *Neurosci Biobehav R* 29(8):1243-1253.

Bolivar, V. J., B. J. Caldarone, A. A. Reilly, and L. Flaherty. 2000. Habituation of activity in an open field: A survey of inbred strains and F1 hybrids. *Behav Genet* 30(4):285-293.

Bomholt, S. F., J. D. Mikkelsen, and G. Blackburn-Munro. 2005. Normal hypothalamo-pituitary-adrenal axis function in a rat model of peripheral neuropathic pain. *Brain Res* 1044(2):216-226.

Bouwknecht, J. A. and R. Paylor. 2002. Behavioral and physiological mouse assays for anxiety: A survey in nine mouse strains. *Behav Brain Res* 136(2):489-501.

Broida, J. and B. Svare. 1982. Strain typical patterns of pregnancy-induced nestbuilding in mice: Maternal and experiential influences. *Physiol Behav* 25:153-157.

Bronson, F. H. 1979. The reproductive ecology of the house mouse. *Quart Rev Biol* 54: 265-299.

Canteras, N. S. 2002. The medial hypothalamic defensive system: Hodological organization and functional implications. *Pharmacol Biochem Behav* 71(3):481-491.

Coe, C. L., G. R. Lubach, W. B. Ershler, and R. G. Klopp. 1989. Influence of early rearing on lymphocyte proliferation response s in juvenile rhesus monkeys. *Brain Behav Immun* 3(1):47-60.

Crawley, J. N. 2000. *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*. New York: Wiley-Liss, John Wiley & Sons, Inc.

Crawley, J. N., J. K. Belknap, A. Collins, J. C. Crabbe, W. Frankel, N. Henderson, R. J. Hitzemann, S. C. Maxson, L. L. Miner, A. J. Silva, J. M. Wehner, A. Wynshaw-Boris, and R. Paylor. 1997. Behavioral phenotypes of inbred mouse strains: Implications and recommendations for molecular studies. *Psychopharmacology* 132(2):107-124.

Dalla, C., K. Antoniou, G. Drossopoulou, M. Xagoraris, N. Kokras, A. Sfikakis, and Z. Papadopoulou-Daifoti. 2005. Chronic mild stress impact: Are females more vulnerable? *Neuroscience* 135(3):703-714.

Dantzer, R. 1986. Behavioral, physiological, and functional aspects of stereotyped behavior: A review and reinterpretation. *J Anim Sci* 62(6):1776-1786.

Dantzer, R. 2004. Cytokine-induced sickness behaviour: A neuroimmune response to activation of innate immunity. *Euro J Pharmacol* 500(1-3):399-411.

Davenport, M. D., S. Tiefenbacher, C. K. Lutz, M. A. Novak, and J. S. Meyer. 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen Comp Endocr* 147(3):255-261.

Davis, M. and P. J. Whalen. 2001. The amygdala: Vigilance and emotion. *Mol Psychiat* 6(1):13-24.

Dawkins, M. S. 1990. From an animal's point of view: Motivation, fitness, and animal welfare. *Behav Brain Sci* 13(1):1-61.

Deak, T., K. T. Nguyen, M. Fleshner, L. R. Watkins, and S. F. Maier. 1999. Acute stress may facilitate recovery from a subcutaneous bacterial challenge. *Neuroimmunomodulation* 6(5):344-354.

Decker, M. W., M. A. Pelleymounter, and M. Gallagher. 1988. Effects of training on a spatial memory task on high affinity choline uptake in hippocampus and cortex in young adult and aged rats. *J Neurosci* 8(1):90-99.

Dhabar, F. S. and K. Viswanathan. 2005. Short-term stress experienced at time of immunization induces a long lasting increase in immunological memory. *Am J Physiol Reg-I* 289: R738-R744.

Draper, W. A. and I. S. Bernstein. 1963. Stereotyped behavior and cage size. *Percept Motor Skill* 16:231-234.

Ducottet, C. and C. Belzung. 2005. Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: Evidence from inbred strains of mice. *Behav Brain Res* 156(1):153-162.

Felten, D. L., S. Y. Felten, S. L. Carlson, J. A. Olschowka, and S. Livnat. 1985. Noradrenergic and peptidergic innervation of lymphoid tissue. *J Immunol* 135(2 Suppl):755-765.

Figueiredo, H. F., C. M. Dolgas, and J. P. Herman. 2002. Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. *Endocrinology* 143(7):2534-2540.

Fischer, W., K. S. Chen, F. H. Gage, and A. Bjorklund. 1992. Progressive decline in spatial learning and integrity of forebrain cholinergic neurons in rats during aging. *Neurobiol Aging* 13(1):9-23.

Fish, E. W., D. Shahrokh, R. Bagot, C. Caldji, T. Bredy, M. Szyf, and M. J. Meaney. 2004. Epigenetic programming of stress responses through variations in maternal care. *Ann NY Acad Sci* 1036:167-180.

Fleshner, M., J. Nermann, L. Lockwood, M. L. Laudenslager, L. R. Watkins, and S. F. Maier. 1995. Stressed rats fail to expand the CD45RC+CD4+ (th1-like) T cell subset in response to KLH: Possible involvement of interferon gamma. *Brain Behav Immun* 9(2):101-112.

Fleshner, M., K. T. Nguyen, C. S. Cotter, L. R. Watkins, and S. F. Maier. 1998. Acute stressor exposure both suppresses acquired immunity and potentiates innate immunity. *Am J Physiol* 275(3 Pt 2):R870-R878.

Fortman, J. D., T. A. Newett, and R. T. Bennett. 2002. *The Laboratory Nonhuman Primate*. Boca Raton, FL: CRC Press.

Fox, J. G., L. C. Anderson, F. M. Loew, and F. W. Quimby, eds. 2002. *Laboratory Animal Medicine,* 2nd ed. San Diego: Academic Press.

Francis, D. D., F. A. Champagne, D. Liu, and M. J. Meaney. 1999. Maternal care, gene expression, and the development of individual differences in stress reactivity. *Ann NY Acad Sci* 896:66-84.

Frick, K. M., L. A. Burlingame, J. A. Arters, and J. Berger-Sweeney. 2000. Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. *Neuroscience* 95(1):293-307.

Garner, J. P. 2006. Perseveration and stereotypy: Systems-level insights from clinical psychology. In *Stereotypic Animal Behavior: Fundamentals and Applications to Welfare, 2nd ed,* G. Mason and J. Rushen, eds. Wallingford, UK: CAB International. 121-153 pp.

Garner, J. P. and G. Mason. 2002. Evidence for a relationship between cage stereotypies and behavioural disinhibition in laboratory rodents. *Behav Brain Res* 136(1):83-92.

Garner, J. P., C. L. Meehan, and J. A. Mench. 2003. Stereotypies in caged parrots, schizophrenia and autism: Evidence for a common mechanism. *Behav Brain Res* 145(1-2):125-134.

Garner, J. P., S. M. Weisker, B. Dufour, and J. A. Mench. 2004. Barbering (fur and whisker trimming) by laboratory mice as a model of human trichotillomania and obsessive-compulsive spectrum disorder. *Comparative Med* 54(2):216-224.

Goto, S., R. Takahashi, S. Araki, and H. Nakamoto. 2002. Dietary restriction initiated in late adulthood can reverse age-related alterations of protein and protein metabolism. *Ann NY Acad Sci* 959:50-56.

Guettinger, H. R. 1979. The integration of learnt and genetically programmed behaviour: A study of hierarchical organization in songs of canaries, greenfinches and their hybrids. *Z Tierpsychol* 49:285-303.

Hawkins, R. K., H. G. Jansen, and S. Sanyal. 1985. Development and degeneration of retina in rds mutant mice: Photoreceptor abnormalities in the heterozygotes. *Exp Eye Res* 41(6):701-720.

Heiderstadt, K. M., R. M. McLaughlin, D. C. Wright, S. E. Walker, and C. E. Gomez-Sanchez. 2000. The effect of chronic food and water restriction on open-filed behaviour and serum corticosterone levels in rats. *Lab Anim* 34(1):20-28.

Heilig, M. 2004. The NPY system in stress, anxiety, and depression. *Neuropeptides* 38(4):213-224.

Herman, J. P. and W. E. Cullinan. 1997. Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 20(2):78-84.

Higley, J. D., S. J. Suomi, and M. Linnoila. 1991. CSF monoamine metabolite concentrations vary according to age, rearing, and sex, and are influenced by the stressor of social separation in rhesus monkeys. *Psychopharmacology (Berl)* 103(4):551-556.

Hubrecht, R. C. 1997. Comfortable quarters for laboratory dogs. In: *Comfortable Quarters for Laboratory Animals*, Viktor Reinhardt, ed. Washington: Animal Welfare Institute. 63-74 pp.

Hubrecht, R. C., J. A. Serpell, and T. B. Poole. 1992. Correlates of pen size and housing conditions on the behaviour of kennelled dogs. *Appl Anim Behav Sci* 34:365-383.

Ichise, M., D. C. Vines, T. Gura, G. M. Anderson, S. J. Suomi, J. D. Higley, and R. B. Innis. 2006. Effects of early life stress on [11C]DASB positron emission tomography imaging of serotonin transporters in adolescent peer- and mother-reared rhesus monkeys. *J Neurosci* 26(17):4638-4643.

Ingram, D. K. 2000. Age-related decline in physical activity: Generalization to nonhumans. *Med Sci Sports Exerc* 32(9):1623-1629.

Ingram, D. K., E. D. London, M. A. Reynolds, S. B. Waller, and C. L. Goodrick. 1981. Differential effects of age on motor performance in two mouse strains. *Neurobiol Aging* 2(3):221-227.

Ingram, D. K., J. Young, and J. A. Mattison. 2007. Calorie restriction in nonhuman primates: Assessing effects on brain and behavioral aging. *Neuroscience* 145(4):1359-1364.

Johnson, R. P., Y. L. Yang, and C. J. Gordon. 2002. Peripheral cholinergic pathway modulates hyperthermia induced by stress in rats exposed to open-field stress. *J Appl Physiol* 92(1):789-794.

Johnson, R. R., T. W. Prentice, P. Bridegam, C. R. Young, A. J. Steelman, T. H. Welsh, C. J. Welsh, and M. W. Meagher. 2006. Social stress alters the severity and onset of the chronic phase of Theiler's virus infection. *J Neuroimmunol* 175(1-2):39-51.

Jordan, T. C., M. B. Hennessy, C. A. Gonzalez, and S. Levine. 1985. Social and environmental factors influencing mother-infant separation-reunion in squirrel monkeys. *Physiol Behav* 34(4):489-493.

Keay, K. A. and R. Bandler. 2002. Parallel circuits mediating distinct emotional coping reactions to different types of stress. *Neurosci Biobehav R* 25(7-8):669-678.

Kempermann, G., D. Gast, and F. H. Gage. 2002. Neuroplasticity in old age: Sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann Neurol* 52(2):135-143.

Kendrick, K. M., M. A. Haupt, M. R. Hinton, K. D. Broad, and J. D. Skinner. 2001. Sex differences in the influence of mothers on the sociosexual preferences of their offspring. *Horm Behav* 40(2):322-338.

Kliethermes, C. L. and J. C. Crabbe. 2006. Genetic independence of mouse measures of some aspects of novelty seeking. *Proc Natl Acad Sci USA* 103(13):5018-5023.

Krief, S., A. Jamart, and C. M. Hladik. 2004. On the possible adaptive value of coprophagy in free-ranging chimpanzees. *Primates* 45(2):141-145.

Krohn, T.C., J. Ritskes-Hoitinga, and P. Svendsen. 1999. The effects of feeding and housing on the behaviour of the laboratory rabbit. *Lab Anim* 33(2):101-107.

Kunkele, J. 1992. Infanticide in wild rabbits *(Oryctolagus cuniculus). J Mammal* 73(2):317-320.

Lamberty, Y. and A. J. Gower. 1992. Age-related changes in spontaneous behavior and learning in NMRI mice from middle to old age. *Physiol Behav* 51(1):81-88.

Latham, N. and G. Mason. 2004. From house mouse to mouse house: The behavioural biology of free-living *Mus musculus* and its implications in the laboratory. *Appl Anim Behav* 86(3-4):261-289.

Laudenslager, M. L., M. Reite, and R. J. Harbeck. 1982. Suppressed immune response in infant monkeys associated with maternal separation. *Behav Neural Biol* 36(1):40-48.

Laudenslager, M. L., P. E. Held, M. L. Boccia, M. L. Reite, and J. J. Cohen. 1990. Behavioral and immunological consequences of brief mother-infant separation: A species comparison. *Dev Psychobiol* 23(3):247-264.

Levine, S. 2005. Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology* 30(10):939-946.

Levine, S. and T. Mody. 2003. The long-term psychobiological consequences of intermittent postnatal separation in the squirrel monkey. *Neuroscience Biobehav R* 27(1-2):83-89.

Levine, S., S. G. Wiener, and C. L. Coe. 1993. Temporal and social factors influencing behavioral and hormonal responses to separation in mother and infant squirrel monkeys. *Psychoneuroendocrinology* 18(4):297-306.

Lewis, M. H., M. F. Presti, J. B. Lewis, and C. A. Turner. The neurobiology of stereotypy I: Environmental complexity. In *Stereotypic Animal Behavior: Fundamentals and Applications to Welfare,* 2nd ed, G. Mason and J. Rushen, eds. Wallingford, UK: CAB International. 196 pp.

Lewis, M. H., Y. Tanimura, L. W. Lee, and J. W. Bodfish. 2007. Animal models of restricted repetitive behavior in autism. *Behav Brain Res* 176(1):66-74.

Li, S. G., J. E. Lawler, D. C. Randall, and D. R. Brown. 1997. Sympathetic nervous activity and arterial pressure responses during rest and acute behavioral stress in SHR versus WKY rats. *J Autonom Nerv Syst* 62(3):147-154.

Lowry, C. A. 2002. Functional subsets of serotonergic neurons: Implications for control of the HPA axis. *J Neuroendocrinol* 14(11):911-923.

Lubach, G. R., C. L. Coe, and W. B. Ershler. 1995. Effects of early rearing environment on immune responses on infant rhesus monkeys. *Brain Behav Imm* 9(1):31-46.

Lutz, C., S. Tiefenbacher, M. Jorgensen, J. Meyer, and M. A. Novak. 2000. Techniques for collecting saliva from awake, unrestrained, adult macaque monkeys for cortisol assay. *Am J Primatol* 52(2):93-99.

Maier, S. F. and L. R. Watkins. 1998. Cytokines for psychologists: Implications of bi-directional immune-to-brain communication for understanding behavior, mood, and thought. *Psychol Rev* 105(1):83-107.

Mangiarini, L., K. Sathasivam, M. Seller, B. Cozens, A. Harper, C. Hetherington, M. Lawton, Y. Trottier, H. Lehrach, S. W. Davies, and G. P. Bates. 1996. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87(3):493-506.

Mason, G. 1991. Stereotypies: A critical review. *Anim Behav* 41(6): 1015-1037.

Mason, G. 2006. Stereotypic behavior in captive animals: Fundamentals and implications for welfare and beyond. In *Stereotypic Animal Behavior: Fundamentals and Applications to Welfare,* 2nd ed, G. Mason and J. Rushen, eds. Wallingford, UK: CAB International. 325-357 pp.

Mason, G. and M. Mendl. 1997. Do the stereotypies of pigs, chickens and mink reflect adaptive species differences in the control of foraging? *Appl Anim Behav Sci*: 53(1-2):45-58.

Mason, G. J. and N. R. Latham. 2004. Can't stop, won't stop: Is stereotypy a reliable animal welfare indicator? *Anim Welf* 13(Suppl):557-569.

McCormick, C. M., J. W. Smythe, S. Sharma, and M. J. Meaney. 1995. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. *Dev Brain Res* 84(1):55-61.

McLaren, G. W., D. W. Macdonald, C. Georgiou, F. Mathews, C. Newman, and R. Mian. 2003. Leukocyte coping capacity: A novel technique for measuring the stress response in vertebrates. *Exp Physiol* 88(4):541-546.

Meaney, M. J., J. Diorio, D. Francis, J. Widdowson, P. LaPlante, C. Caldji, S. Sharma, J. R. Seckl, and P. M. Plotsky. 1996. Early environmental regulation of forebrain glucocorticoid receptor gene expression: Implications for adrenocortical responses to stress. *Dev Neurosci* 18(1-2):49-72.

Miller, J. B. and J. P. O'Callaghan. 2002. Neuroendocrine aspects of the response to stress. *Metabolism* 51(6 Suppl):5-10.

Mineur, Y. S., D. J. Prasol, C. Belzung, and W. E. Crusio. 2003. Agonistic behavior and unpredictable chronic mild stress in mice. *Behav Genet* 33(5):513-519.

Morton, D. B. 2002. Behaviour of rabbits and rodents. In *The Ethology of Domestic Animals—An Introductory Text,* P. Jensen, ed. Oxford: CABI Wallingford Oxford. 193-209 pp.

Morton, D. B. and P. H. M. Griffiths. 1985. Guidelines on the recognition of pain, distress, and discomfort in experimental animals and an hypothesis for assessment. *Vet Record* 116(16):431-436.

Moy, S. S., J. J. Nadler, A. Perez, R. P. Barbaro, J. M. Johns, T. R. Magnuson, J. Piven, and J. N. Crawley. 2004. Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes Brain Behav* 3(5):287-302.

Muglia, L., L. Jacobson, P. Dikkes, and J. A. Majzoub. 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature* 373(6513):427-432.

Nash, L. T., J. Fritz, P. A. Alford, and L. Brent. 1999. Variables influencing the origins of diverse abnormal behaviors in a large sample of captive chimpanzees (Pan troglodytes). *Am J Primatol* 48(1):15-29.

Nemeroff, C.B. and W. W. Vale. 2005. The neurobiology of depression: Inroads to treatment and new drug discovery. *J Clin Psychiat* 66(Suppl 7):5-13.

Novak, M. A. 2003. Self-injurious behavior in rhesus monkeys: New insights into its etiology, physiology, and treatment. *Am J Primatol* 59(1):3-19.

Novak, M. A. and S. J. Suomi. 1988. Psychological well-being of primates in captivity. *Am Psychol* 43(10):765-73.

O'Connor, K. A., J. D. Johnson, M. K. Hansen, J. L. Wiesler-Frank, L. R. Watkins, and S. F. Maier. 2003. Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Res* 991:123-132.

Olsson, I. A. S. and C. M. Sherwin. 2006. Behaviour of laboratory mice in different housing conditions when allowed to self-administer an anxiolytic. *Lab Anim* 40(4):392-399.

Parker, K. J., C. L. Buckmaster, K. Sundlass, A. F. Schatzberg, and D. M. Lyons. 2006. Maternal mediation, stress inoculation, and the development of neuroendocrine stress resistance in primates. *Proc Nat Acad Sci USA* 103(8):3000-3005.

Pezze, M. A. and J. Feldon. 2004. Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol* 74(5):301-320.

Randall, D. C., D. R. Brown, L. V. Brown, and J. M. Kilgore. 1994. Sympathetic nervous activity and arterial blood pressure control in conscious rat during rest and behavioral stress. *Am J Physiol* 267(5 Part 2):R1241-R1249.

Ribeiro, S. C., S. E. Kennedy, Y. R. Smith, C. S. Stohler, and J. K. Zubieta. 2005. Interface of physical and emotional stress regulation through the endogenous opioid system and mu-opioid receptors. *Prog Neuropsychopharmacol Biol Psychiatry* 29(8):1264-1280.

Ruppenthal, G. C., C. G. Walker, and G. P. Sackett. 1991. Rearing infant monkeys *(Macaca nemestrina)* in pairs produces deficient social development compared with rearing in single cages. *Am J Primatol* 25(2):103-113.

Ruppenthal, G. C., M. K. Harlow, C. D. Eisele, H. F. Harlow, and S. J. Suomi. 1974. Development of peer interactions of monkeys reared in a nuclear-family environment. *Child Dev* 45(3):670-682.

Sackett, G. P., G. C. Ruppenthal, and A. E. Davis. 2002. Survival, growth, health, and reproduction following nursery rearing compared with mother rearing in pigtailed monkeys *(Macaca nemestrina)*. *Am J Primatol* 56(3):165-183.

Sanders, V. M., D. J. Kasprowicz, A. P. Kohm, and M. A. Swanson. 2001. Neurotransmitter receptors on lymphocytes and other lymphoid cells. In *Psychoneuroimmunology*, 3rd ed. R. Ader, D. L. Felten, and N. Cohen, eds. New York: Academic Press. 161-197 pp.

Serrats, J. and P. E. Sawchenko. 2006. CNS activational responses to staphylococcal enterotoxin B: T-lymphocyte-dependent immune challenge effects on stress-related circuitry. *J Comp Neurol* 495(2):236-254.

Sherwin, C. 1998. Voluntary wheel running: A review and novel interpretation. *Anim Behav* 56(1):11-27.

Silver, L. M. 1995. *Mouse Genetics*: *Concepts and Applications*. New York: Oxford University Press.

Smith, L. K. and G. A. Metz. 2005. Dietary restriction alters fine motor function in rats. *Physiol Behav* 85(5):581-592.

Snowdon, C. T. and A. Savage. 1989. Psychological well-being of captive primates: General considerations and examples from callitrichids. In *Housing, Care and Psychological Wellbeing of Captive and Laboratory Primates*, E. F. Segal, ed. Park Ridge, NJ: Noyes Publications. 75-88 pp.

Sotres-Bayon, F., C. F. Cain, and J. E. LeDoux. 2006. Brain mechanisms of fear extinction: Historical perspectives on the contributions of prefrontal cortex. *Biol Psychiat* 60(4):329-336.

Stenzel-Poore, M., S. C. Heinrichs, S. Rivest, G. F. Koob, and W. W. Vale. 1994. Overproduction of corticotrophin-releasing factor in transgenic mice: A genetic model of anxiogenic behavior. *J Neurosci* 14(5 Pt 1):2579-2584.

Suomi, S. J. 2004. How gene-environment interactions shape biobehavioral development: Lessons from studies with rhesus monkeys. *Res Hum* 1:205-222.

Thomas, G. 2002. The S6 kinase signaling pathway in the control of development and growth. *Biol Res* 35(2):305-313.

Tiefenbacher, S. T., M. A. Novak, C. K. Lutz, and J. S. Meyer. 2005. The physiology and neurochemistry of self-injurious behavior: A nonhuman primate model. *Front Biosci* 10(1):1-11.

Tournier, J. N., J. Mathieu, Y. Mailfert, E. Multon, C. Drouet, A. Jouan, and E. Drouet. 2001. Chronic restraint stress induces severe disruption of the T-cell specific response to tetanus toxin vaccine. *Immunology* 102(1):515-523.

Ulrich-Lai, Y. M, W. Xie, J. T. Meij, C. M. Dolgas, L. Yu, and J. P. Herman. 2006. Limbic and HPA axis function in an animal model of chronic neuropathic pain. *Physiol Behav* 88(1-2):67-76.

van Bogaert, M. J., L. Groenink, R. S. Oosting, K. G. Westphal, J. van der Gugten, and B. Olivier. 2006. Mouse strain differences in autonomic responses to stress. *Genes Brain Behav* 5(2):139-149.

van Praag, H., G. Kempermann, and F. H. Gage. 2000. Neural consequences of environmental enrichment. *Nat Rev Neurosci* 1(3):191-198.

Vanderschuren, L. J., R. J. Niesink, and J. M. Van Ree. 1997. The neurobiology of social play behavior in rats. *Neurosci Biobehav R* 21(3):309-326.

Vogt, J. L., C. L. Coe, E. Lowe, and S. Levine. 1980. Behavioral and pituitary-adrenal response of adult squirrel monkeys to mother-infant separation. *Psychoneuroendocrinology* 5(3):181-190.

Wallace, C. S., V. L. Kilman, G. S. Withers, and W. T. Greenough. 1992. Increases in dendritic length in occipital cortex after 4 days of differential housing in weanling rats. *Behav Neural Biol* 58(1):64-68.

Weaver, I. C. G., M. J. Meaney, and M. Szyf. 2006. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proc Natl Acad Sci USA* 103(9):3480-3485.

Weaver, I. C. G., N. Cervoni, F. A. Champagne, A. C. D'Alessio, S. Sharma, J. R. Seckl, S. Dymov, M. Szyf, and M. J. Meaney. 2004. Epigenetic programming by maternal behavior. *Nat Neurosci* 7:847-854.

Windle, R. J., S. A. Wood, N. Shanks, S. L. Lightman, and C. D. Ingram. 1998b. Ultradian rhythm of basal corticosterone release in the female rat: Dynamic interaction with the response to acute stress. *Endocrinology* 139(2):443-450.

Wolfer, D. P., O. Litvin, S. Morf, R. M. Nitsch, H. P. Lipp, and H. Würbel. 2004. Cage enrichment and mouse behavior. *Nature* 432(7019):821-822.

Würbel, H. 2001. Ideal homes? Housing effects on rodent brain and behaviour. *Trends Neurosci* 24(4):207-211.

Zhang, W. and P. Thoren. 1998. Hyper-responsiveness of adrenal sympathetic nerve activity in spontaneously hypertensive rats to ganglionic blockade, mental stress and neuronglucopenia. *Pflugers Arch* 437(1):56-60.

ADDITIONAL REFERENCES

Page 26 The identification of species-typical behavior often comes from ethograms developed by researchers to describe the kinds of behavior that animals display in various settings (Bronson 1979).

Chopra, P. K., P. K. Seth, and S. Seth. 1992. Behavioural profile of free-ranging rhesus monkeys. *Primate Rep* 32:75-105.

Latham, N. and G. Mason. 2004. From house mouse to mouse house: The behavioural biology of free-living *Mus musculus* and its implications in the laboratory. *Appl Anim Behav* 86(3-4):261-289.

Mayer, J. 2007. Use of behavior analysis to recognize pain in small animals. *Lab Anim* 36(6):43-48.

Morton, D. B. 2002. Behaviour of rabbits and rodents. In *The Ethology of Domestic Animals—An Introductory Text,* Per Jensen, ed. Oxford: CABI Wallingford Oxford. 193-209 pp.

Morton, D. B. and P. H. M. Griffiths. 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Record* 116(16):431-436.

Olivier B. and D. van Dalen. 1982. Social behaviour in rats and mice: An ethologically based model for differentiating psychoactive drugs. *Aggress Behav* 8(2):163-168.

Page 27 Such age-related changes (e.g., hearing and vision deficits) have been documented for a number of murine strains (Hawkins et al. 1985).

Johnson, K. R., Q. Y. Zheng, and K. Noben-Trauth. 2006. Strain background effects and genetic modifiers of hearing in mice. *Brain Res* 1091(1):79-88.

Jones, S. M., T. A. Jones, K. R. Johnson, H. Yu, L. C. Erway, and Q. Y. Zheng. 2006. A comparison of vestibular and auditory phenotypes in inbred mouse strains. *Brain Res* 1091(1):40-46.

Malek, G., L. V. Johnson, B. E. Mace, P. Saloupis, D. E. Schmechel, D. W. Rickman, C. A. Toth, P. M. Sullivan, and C. Bowes Rickman. 2005. Apolipoprotein E allele-dependent pathogenesis: A model for age-related retinal degeneration. *Proc Natl Acad Sci USA* 102(33):11900-11905.

Ohlemiller, K. K. 2006. Contributions of mouse models to understanding of age- and noise-related hearing loss. *Brain Res* 1091(1):89-102.

Smith, R. S., S. W. John, A. Zabeleta, M. T. Davisson, N. L. Hawes, and B. Chang. 2000. The bst locus on mouse chromosome 16 is associated with age-related subretinal neovascularization. *Proc Natl Acad Sci USA* 97(5):2191-2195.

Page 28 In fact, inbred strains of mice differ in almost every behavioral, sensory, motor, and physiological trait studied to date, such as anxiety, learning and memory, brain structure and size, visual acuity, acoustic startle, exploratory behavior, alcohol sensitivity, depression, pain sensitivity, and motor coordination (Crawley et al. 2000).

Bolivar, V. J., B. J. Caldarone, A. A. Reilly, and L. Flaherty. 2000. Habituation of activity in an open field: A survey of inbred strains and F1 hybrids. *Behav Genet* 30(4):285-293.

Bolivar, V. J., O. Pooler, and L. Flaherty. 2001. Inbred strain variation in contextual and cued fear conditioning behavior. *Mamm Genome* 12(8):651-656.

Bothe, G. W. M., V. J. Bolivar, M. J. Vedder, and J. G. Geistfeld. 2005. Behavioral differences among fourteen inbred mouse strains commonly used as disease models. *Comparative Med* 55(4):326-334.

Bouwknecht, J. A. and R. Paylor. 2002. Behavioral and physiological mouse assays for anxiety: A survey in nine mouse strains. *Behav Brain Res* 136(2):489-501.

Cook, M. N., R. W. Williams, and L. Flaherty. 2001. Anxiety-related behaviors in the elevated zero-maze are affected by genetic factors and retinal degeneration. *Behav Neurosci* 115(2):468-476.

Crabbe, J. C., D. Wahlsten, and B. C. Dudek. 1999. Genetics of mouse behavior: Interactions with laboratory environment. *Science* 284(5420):1670-1672.

Crabbe, J. C., P. Metten, I. Ponomarev, C. A. Prescott, and D. Wahlsten. 2006. Effects of genetic and procedural variation on measurement of alcohol sensitivity in mouse inbred strains. *Behav Genet* 36(4):536-552.

Guillot, P. V., P. L. Roubertoux, and W. E. Crusio. 1994. Hippocampal mossy fiber distributions and intermale aggression in seven inbred mouse strains. *Brain Res* 660(1):167-169.

Kliethermes, C. L. and J. C. Crabbe. 2006. Genetic independence of mouse measures of some aspects of novelty seeking. *Proc Natl Acad Sci USA* 103(13):5018-5023.

Liu, X. and H. K. Gershenfeld. 2001. Genetic differences in the tail-suspension test and its relationship to imipramine response among 11 inbred strains of mice. *Biol Psychiat* 49(7):575-581.

Logue, S. F., E. H. Owen, D. L. Rasmussen, and J. M. Wehner. 1997. Assessment of locomotor activity, acoustic and tactile startle, and prepulse inhibition of startle in inbred mouse strains and F1 hybrids: Implications of genetic background for single gene and qualitative trait loci analyses. *Neuroscience* 80(4):1075-1086.

Lucki, I., A. Dalvi, and A. J. Mayorga. 2001. Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology* 155(3):315-322.

McFadyen, M. P., G. Kusek, V. J. Bolivar, and L. Flaherty. 2003. Differences among eight inbred strains of mice in motor ability and motor learning on a rotorod. *Genes Brain Behav* 2(4):214-219.

Mogil, J. S., S. G. Wilson, K. Bon, S. E. Lee, K. Chung, P. Raber, J. O. Pieper, H. S. Hain, J. K. Belknap, L. Hubert, G. I. Elmer, J. M. Chung, and M. Devor. 1999. Heritability of nociception I: Responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 80(1-2):67-82.

Moy, S. S., J. J. Nadler, A. Perez, R. P. Barbaro, J. M. Johns, T. R. Magnuson, J. Piven, and J. N. Crawley. 2004. Sociability and preference for social novelty in five inbred strains: An approach to assess autistic-like behavior in mice. *Genes Brain Behav* 3(5):287-302.

Owen, E. H., S. F. Logue, D. L. Rasmussen, and J. M. Wehner. 1997. Assessment of learning by the Morris water task and fear conditioning in inbred mouse strains and F1 hybrids: Implications of genetic background for single gene mutations and quantitative trait loci analyses. *Neuroscience* 80(4):1087-1099.

Rustay, N. R., D. Wahlsten, and J. C. Crabbe. 2003. Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. *Behav Brain Res* 141(2):237-249.

Trullas, R. and P. Skolnick. 1993. Differences in fear motivated behaviors among inbred mouse strains. *Psychopharmacology* 111(3):323-331.

van Bogaert, M. J., L. Groenink, R. S. Oosting, K. G. Westphal, J. van der Gugten, and B. Olivier. 2006. Mouse strain differences in autonomic responses to stress. *Genes Brain Behav* 5(2):139-149.

Wahlsten, D., P. Metten, and J. C. Crabbe. 2003a. A rating scale for wildness and ease of handling laboratory mice: Results for 21 inbred strains tested in two laboratories. *Genes Brain Behav* 2(2):71-79.

Wahlsten, D., P. Metten, and J. C. Crabbe. 2003b. Survey of 21 inbred mouse strains in two laboratories reveals that BTBR T/+ tf/tf has severely reduced hippocampal commissure and absent corpus callosum. *Brain Res* 971(1):47-54.

Wahlsten, D., S. F. Cooper, and J. C. Crabbe. 2005. Different rankings of inbred mouse strains on the Morris maze and a refined 4-arm water escape task. *Behav Brain Res* 165(1):36-51.

Willott, J. F., L. Tanner, J. O'Steen, K. R. Johnson, M. A. Bogue, and L. Gagnon. 2003. Acoustic startle and prepulse inhibition in 40 inbred strains of mice. *Behav Neurosci* 117(4):716-727.

Wong, A. A. and R. E. Brown. 2005. Visual detection, pattern discrimination and visual acuity in 14 inbred strains. *Genes Brain Behav* 5(5):389-403.

Page 28 For instance, inbred strains differ in performance on anxiety, depression, and fear learning assays (Balogh and Wehner 2003).

Bolivar, V. J., O. Pooler, and L. Flaherty. 2001. Inbred strain variation in contextual and cued fear conditioning behavior. *Mamm Genome* 12(8):651-656.

Bouwknecht, J. A. and R. Paylor. 2002. Behavioral and physiological mouse assays for anxiety: A survey in nine mouse strains. *Behav Brain Res* 136(2):489-501.

Cook, M. N., R. W. Williams, and L. Flaherty. 2001. Anxiety-related behaviors in the elevated zero-maze are affected by genetic factors and retinal degeneration. *Behav Neurosci* 115(2):468-476.

Trullas, R. and P. Skolnick. 1993. Differences in fear motivated behaviors among inbred mouse strains. *Psychopharmacology* 111(3):323-331.

Page 32 Environments that elicit or enhance stereotypies not as part of defined pathophysiology or disease models are typically suboptimal (Berkson and Mason 1964).

Garner, J. P. and G. Mason. 2002. Evidence for a relationship between cage stereotypies and behavioural disinhibition in laboratory rodents. *Behav Brain Res* 136(1):83-92.

Garner, J. P., C. L. Meehan, and J. A. Mench. 2003. Stereotypies in caged parrots, schizophrenia and autism: Evidence for a common mechanism. *Behav Brain Res* 145(1-2):125-134.

Mason, G. 1991. Stereotypies: A critical review. *Anim Behav* 41(6):1015-1037.

Mason, G. J. and N. R. Latham. 2004. Can't stop, won't stop: Is stereotypy a reliable animal welfare indicator? *Anim Welf* 13(Suppl):557-569.

Ridley, R. M. and H. F. Baker. 1982. Stereotypy in monkeys and humans. *Psychol Med* 12(1):61-72.

Würbel, H. 2001. Ideal homes? Housing effects on rodent brain and behaviour. *Trends Neurosci* 24(4):207-211.

Page 32 Classic whole-body stereotypies include circling, pacing (dogs, primates), wall bouncing (dogs), and somersaulting and bar chewing (rodents), whereas self- or other-animal-directed stereotypies often involve the limbs and/or face and include such patterns as digit sucking, paw licking, and overgrooming (Bayne and Novak 1998).

Callard, M. D., S. N. Bursten, and E. O. Price. 2000. Repetitive backflipping behaviour in captive roof rats (*Rattus rattus*) and the effects of cage enrichment. *Anim Welf* 9(2):139-152.

Garner, J. P. 2005. Stereotypies and other abnormal repetitive behaviors: Potential impact on validity, reliability, and replicability of scientific outcomes. *ILAR J* 46(2):106-117.

Hubrecht, R. C., J. A. Serpell, and T. B. Poole. 1992. Correlates of pen size and housing conditions on the behaviour of kennelled dogs. *Appl Anim Behav Sci* 34:365-383.

Luescher, U. A., D. B. McKeown, and J. Halip. 1991. Stereotypic or obsessive-compulsive disorders in dogs and cats. *Vet Clin North Am-Small* 21(2):401-413.

Morton, D. B., M. Jennings, G. R. Batchelor, D. Bell, L. Birke, K. Davies, J. R. Eveleigh, D. Gunn, M. Heath, B. Howard, P. Koder, J. Phillips, T. Poole, A. W. Sainsbury, G. Sales, D. J. A. Smith, M. Stauffacher, and R. J. Turner. 1993. Refinements in rabbit husbandry. *Lab Anim* 27(4):301-329.

Waiblinger, E. and B. Konig. 2004. Refinement of gerbil housing and husbandry in the laboratory. *Anim Welf* 13(Suppl):S229-S235.

Page 34 Such protocols are widely used in gerontology research where diet has been shown to slow aging, extend lifespan, and reduce the incidence of age-related diseases in rodents (Goto et al. 2002).

Goto, S., R. Takahashi, Z. Radak, and R. Sharma. 2007. Beneficial biochemical outcomes of late-onset dietary restriction in rodents. *Ann N Y Acad Sci* 1100:431-441.

Jamieson, H. A., S. N. Hilmer, V. C. Cogger, A. Warren, R. Cheluvappa, D. R. Abernethy, A. V. Everitt, R. Fraser, R. de Cabo, and D. G. Le Couteur. 2007. Caloric restriction reduces age-related pseudocapillarization of the hepatic sinusoid. *Exp Gerontol* 42(4):374-378.

Hyun, D. H., S. S. Emerson, D. G. Jo, M. P. Mattson, and R. de Cabo. 2006. Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. *Proc Natl Acad Sci USA* 103(52):19908-19912.

Seymour, E. M., R. V. Parikh, A. A. Singer, and S. F. Bolling. 2006. Moderate calorie restriction improves cardiac remodeling and diastolic dysfunction in the Dahl-SS rat. *J Mol Cell Cardiol* 41(4):661-668.

Page 36 Types of behavior commonly explored to investigate the presence of stress include open-field activity, movements in an elevated plus maze, changes in innate behaviors (e.g., movement, grooming, feeding, sexual behavior), defensive behaviors (to external threats), and avoidance/escape (Beck and Luine 2002).

Blanchard, R. J., C. R. McKittrick, and D. C. Blanchard. 2001. Animal models of social stress: Effects on behavior and brain neurochemical systems. *Physiol Behav* 73(3):261-271.

Ely, D. R., V. Dapper, J. Marasca, J. B. Correa, G. D. Gamaro, M. H. Xavier, M. B. Michalowski, D. Catelli, R. Rosat, M. B. Ferreira, and C. Dalmaz. 1997. Effect of restraint stress on feeding behavior of rats. *Physiol Behav* 61(3):395-398.

Harkin, A., T. J. Connor, J. M. O'Donnell, and J. P. Kelly. 2002. Physiological and behavioral responses to stress: What does a rat find stressful? *Lab Anim* 31(4):42-50.

Mangiavacchi, S., F. Masi, S. Scheggi, B. Leggio, M. G. De Montis, and C. Gambarana. 2001. Long-term behavioral and neurochemical effects of chronic stress exposure in rats. *J Neurochem* 79(6):1113-1121.

Pare, W. P. and S. M. Tejani-Butt. 1996. Effect of stress on the behavior and 5-HT system in Sprague-Dawley and Wistar Kyoto rat strains. *Integr Physiol Beh Sci* 31(2):112-121.

Perrot-Sinal, T. S., A. Gregus, D. Boudreau, and L. E. Kalynchuk. 2004. Sex and repeated restraint stress interact to affect cat odor-induced defensive behavior in adult rats. *Brain Res* 1027:161-172.

Retana-Marquez, S., E. D. Salazar, and J. Velazquez-Moctezuma. 1996. Effect of acute and chronic stress on masculine sexual behavior in the rat. *Psychoneuroendocrinology* 21(1):39-50.

Rittenhouse, P. A., C. Lopez-Rubalcava, G. D. Stanwood, and I. Lucki. 2002. Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat. *Psychoneuroendocrinology* 27(3):303-318.

Page 37 Glucocorticoids are typically measured in blood serum or plasma but can also be quantified in saliva, urine, feces, and hair (Abelson et al. 2005).

Anderson, S. M., G. A. Saviolakis, R. A. Bauman, K. Y. Chu, S. Ghosh, and G. J. Kant. 1996. Effects of chronic stress on food acquisition, plasma hormones, and the estrous cycle of female rats. *Physiol Behav* 60:325-329.

Atkinson, H. C., S. A. Wood, Y. M. Kershaw, E. Bate, and S. L. Lightman. 2006. Diurnal variation in the responsiveness of the hypothalamic-pituitary-adrenal axis of the male rat to noise stress. *J Neuroendocrinol* 18(7):526-533.

Belz, E. E., J. S. Kennell, R. K. Czambel, R. T. Rubin, and M. E. Rhodes. 2003. Environmental enrichment lowers stress-responsive hormones in singly housed male and female rats. *Pharmacol Biochem Behav* 76(3-4):481-486.

Blanchard, R. J., C. R. McKittrick, and D. C. Blanchard. 2001. Animal models of social stress: Effects on behavior and brain neurochemical systems. *Physiol Behav* 73(3):261-271.

Brown, K. J. and N. E. Grunberg. 1995. Effects of housing on male and female rats: Crowding stresses males but calms females. *Physiol Behav* 58(6):1085-1089.

Cavigelli, S. A., S. L. Monfort, T. K. Whitney, Y. S. Mechref, M. Novotny, and M. K. McClintock. 2005. Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *J Endocrinol* 184(1):153-163.

Culman, J., I. J. Kopin, and J. M. Saavedra. 1991. Regulation of corticotropin-releasing hormone and pituitary-adrenocortical response during acute and repeated stress in the rat. *Endocr Reg* 25(3):151-158.

Davenport, M. D, S. T. Tiefenbacher, C. K. Lutz, M. A. Novak, and J. S. Meyer. 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen Comp Endocr* 147(3):255-261.

De Boer, S. F., J. L. Slangen, and G. J. van der Gugten. 1988. Adaptation of plasma catecholamine and corticosterone responses to short-term repeated noise stress in rats. *Physiol Behav* 44(2):273-280.

De Boer, S. F., S. J. Koopmans, J. L. Slangen, and G. J. van der Gugten. 1990. Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: Effect of interstressor interval length. *Physiol Behav* 47(6):1117-1124.

Dhabhar, F. S., B. S. McEwen, and R. L. Spencer. 1993. Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels—a comparison between Sprague-Dawley, Fischer 344 and Lewis rats. *Brain Res* 616(1-2):89-98.

Donnerer, J. and F. Lembeck. 1990. Different control of the adrenocorticotropin-corticosterone response and of prolactin secretion during cold stress, anesthesia, surgery, and nicotine injection in the rat: Involvement of capsaicin-sensitive sensory neurons. *Endocrinology* 126(2):921-926.

Faraday, M. M., K. H. Blakeman, and N. E. Grunberg. 2005. Strain and sex alter effects of stress and nicotine on feeding, body weight, and HPA axis hormones. *Pharmacol Biochem Be* 80(4):577-589.

Fediuc, S., J. E. Campbell, and M. C. Riddell. 2006. Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Daley rats. *J Appl Physiol* 100(6):1867-1875.

Guo, A. L., F. Petraglia, M. Criscuolo, G. Ficarra, R. E. Nappi, M. Palumbo, A. Valentini, and A. R. Genazzani. 1994. Acute stress- or lipopolysaccharide-induced corticosterone secretion in female rats is independent of the oestrous cycle. *Eur J Endocrinol* 131(5):535-539.

Hall, F. S., J. M. Sundstrom, J. Lerner, and A. Pert. 2001. Enhanced corticosterone release after a modified forced swim test in Fawn hooded rats is independent of rearing experience. *Pharmacol Biochem Be* 69(3-4):629-634.

Harper, J. M. and S. N. Austad. 2000. Fecal glucocorticoids: A noninvasive method of measuring adrenal activity in wild and captive rodents. *Physiol Biochem Zool* 73(1):12-22.

Ishikawa, M., S. Ohdo, H. Watanabe, C. Hara, and N. Ogawa. 1995. Alteration in circadian rhythm of plasma corticosterone in rats following sociopsychological stress induced by communication box. *Physiol Behav* 57(1):41-47.

Kirschbaum, C. and D. H. Hellhammer. 1994. Salivary cortisol in psychoneuroendocrine research: Recent developments and applications. *Psychoneuroendocrinology* 19(4):313-333.

Klosterman, L. L, J. T. Murai, and P. K. Siiteri. 1986. Cortisol levels, binding, and properties of corticosteroid-binding globulin in the serum of primates. *Endocrinology* 118(1):424-434.

Ling, S. and F. Jamali. 2003. Effect of cannulation surgery and restraint stress on the plasma corticosterone concentration in the rat: Application of an improved corticosterone HPLC assay. *J Pharm Pharm Sci* 6(2):246-251.

Livezey, G. T., J. M. Miller, and W. H. Vogel. 1985. Plasma norepinephrine, epinephrine and corticosterone stress responses to restraint in individual male and female rats, and their correlations. *Neurosci Lett* 62(1):51-56.

Lynch, J. W., M. Z. Khan, J. Altmann, M. N. Njahira, and N. Rubenstein. 2003. Concentrations of four fecal steroids in wild baboons: Short-term storage conditions and consequences for data interpretation. *Gen Comp Endocr* 132(2):264-271.

Marti, O., A. Gavalda, T. Jolin, and A. Armario. 1993. Effect of regularity of exposure to chronic immobilization stress on the circadian pattern of pituitary adrenal hormones, growth hormone, and thyroid stimulating hormone in the adult male rat. *Psychoneuroendocrinology* 18(1):67-77.

Moncek, F., R. Duncko, B. B. Johansson, and D. Jezova. 2004. Effect of environmental enrichment on stress related systems in rats. *J Neuroendocrinol* 16(5):423-431.

Orr, T. E., J. L. Meyerhoff, E. H. Mougey, and B. N. Bunnell. 1990. Hyperresponsiveness of the rat neuroendocrine system due to repeated exposure to stress. *Psychoneuroendocrinology* 15(5-6):317-328.

Rivier, C. and W. Vale. 1987. Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: A pituitary-mediated mechanism. *Endocrinology* 121(4):1320-1328.

Royo, F., N. Bjork, H. E. Carlsson, S. Mayo, and J. Hau. 2004. Impact of chronic catheterization and automated blood sampling (Accusampler) on serum corticosterone and fecal immunoreactive corticosterone metabolites and immunoglobulin A in male rats. *J Endocrinol* 180(1):145-153.

Sakellaris, P. C. and J. Vernikos-Danellis. 1975. Increased rate of response of the pituitary-adrenal system in rats adapted to chronic stress. *Endocrinology* 97(3):597-602.

Sapolsky, R., M., L. C. Krey, and B. S. McEwen. 1983. The adrenocortical stress-response in the aged male rat: impairment of recovery from stress. *Exp Gerontol* 18(1):55-64.

Tannenbaum, B. M., W. Rowe, S. Sharma, J. Diorio, A. Steverman, M. Walker, and M. J. Meaney. 1997a. Dynamic variations in plasma corticosteroid-binding globulin and basal HPA activity following acute stress in adult rats. *J Neuroendocrinol* 9(3):163-168.

Tannenbaum, B. M., D. N. Brindley, G. S. Tannenbaum, M. F. Dallman, M. D. McArthur, and M. J. Meaney. 1997b. High-fat feeding alters both basal and stress-induced hypothalamic-pituitary-adrenal activity in the rat. *Am J Physiol* 273(6 Pt 1):E1168-E1177.

Tuli, J., J. A. Smith, and D. B. Morton. 1995a. Corticosterone, adrenal and spleen weight in mice after tail bleeding, and its effect on nearby animals. *Lab Anim* 29(1):90-95.

Tuli, J., J. A. Smith, and D. B. Morton. 1995b. Effects of acute and chronic restraint on the adrenal gland weight and serum corticosterone concentration of mice and their faecal output of oocysts after infection with Eimeria apionoides. *Res Vet Sci* 59(1):82-86.

Tuli, J., J. A. Smith, and D. B. Morton. 1995c. Stress measurements in mice after transportation. *Lab Anim* 29(2):132-138.

Vachon, P. and J. P. Moreau. 2001. Serum corticosterone and blood glucose in rats after two jugular vein blood sampling methods: Comparison of the stress response. *Contemp Top Lab Anim* 40(5):22-24.

Vahl, T. P., Y. M. Ulrich-Lai, M. M. Ostrander, C. M. Dolgas, E. E. Elfers, R. J. Seeley, D. A. D'Alessio, and J. P. Herman. 2005. Comparative analysis of ACTH and corticosterone sampling methods in rats. *Am J Physiol-Endoc M* 289:E823-E828.

Viau, V. and M. J. Meaney. 1991. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* 129(5):2503-2511.

Viveros, M. P., R. Hernandez, I. Martinez, and P. Gonzalez. 1988. Effects of social isolation and crowding upon adrenocortical reactivity and behavior in the rat. *Rev Esp Fisiol* 44(3):315-321.

Weinstock, M., M. Razin, D. Schorer-Apelbaum, D. Men, and R. McCarty. 1998. Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. *Int J Dev Neurosci* 16(3-4):289-295.

Windle, R. J., S. A. Wood, N. Shanks, P. Perks, G. L. Conde, A. P. da Costa, C. D. Ingram, and S. L. Lightman. 1997. Endocrine and behavioural responses to noise stress: Comparison of virgin and lactating female rats during non-disrupted maternal activity. *J Neuroendocrinol* 9(6):407-414.

Windle, R. J., S. A. Wood, S. L. Lightman, and C. D. Ingram. 1998a. The pulsatile characteristics of hypothalamo-pituitary-adrenal activity in female Lewis and Fischer 344 rats and its relationship to differential stress responses. *Endocrinology* 139(10):4044-4052.

Page 38 While some (prolactin, α-MSH, oxytocin) increase during stress, others decrease (growth hormone, luteinizing hormone, prolactin), depending on the animal species and the physiological state in which stress occurs (Armario et al. 1984).

Bingaman, E. W., L. D. Van de Kar, J. M. Yracheta, Q. Li, and T. S. Gray. 1995. Castration attenuates prolactin response but potentiates ACTH response to conditioned stress in the rat. *Am J Physiol* 269(4 Pt 2):R856-R863.

Brown, G. M., D. S. Schalch, and S. Reichlin. 1971. Patterns of growth hormone and cortisol responses to psychological stress in the squirrel monkey. *Endocrinology* 88(4):956-963.

Brown, G. M., J. Seggie, and J. Feldmann. 1977. Effect of psychosocial stimuli and limbic lesions on prolactin at rest and following stress. *Clin Endocrinol* 6(Suppl):29S-41S.

Cates, P. S., M. L. Forsling, and K. T. O'byrne. 1999. Stress-induced suppression of pulsatile luteinising hormone release in the female rat: Role of vasopressin. *J Neuroendocrinol* 11(9):677-683.

Collu, R., J. C. Jequier, J. Letarte, G. Leboeuf, and J. R. Ducharme. 1973. Effect of stress and hypothalamic deafferentation on the secretion of growth hormone in the rat. *Neuroendocrinology* 11(3):183-190.

Cronin, M. T., B. J. Siegel, and G. P. Moberg. 1981. Effect of behavioral stress on plasma levels of growth hormone in sheep. *Physiol Behav* 26(5):887-890.

Day, T. A., M. J. West, and J. O. Willoughby. 1983. Stress suppression of growth hormone secretion in the rat: Effects of disruption of inhibitory noradrenergic afferents to the median eminence. *Aust J Biol Sci* 36(5-6):525-530.

Dunn, J. D., W. J. Schindler, M. D. Hutchins, L. E. Scheving, and C. Turpen. 1973. Daily variation in rat growth hormone concentration and the effect of stress on periodicity. *Neuroendocrinology* 13(1):69-78.

Furudate, S., H. Ashihara, and T. Nakano. 1989. Prolactin secretion and its response to stress during the estrous cycle of the rats. *Jikken Dobutsu* 38(4):313-318.

Gala, R. R. 1990. The physiology and mechanisms of the stress-induced changes in prolactin secretion in the rat. *Life Sci* 46(20):1407-1420.

Gala, R. R. and D. J. Haisenleder. 1986. Restraint stress decreases afternoon plasma prolactin levels in female rats: Influence of neural antagonists and agonists on restraint-induced changes in plasma prolactin and corticosterone. *Neuroendocrinology* 43(2):115-123.

Hashimoto, K., K. Murakami, T. Takao, S. Makino, M. Sugawara, and Z. Ota. 1989. Effect of acute ether or restraint stress on plasma corticotropin-releasing hormone, vasopressin and oxytocin levels in the rat. *Acta Med Okayama* 43(3):161-167.

Husain, M. K., W. M. Manger, T. W. Rock, R. J. Weiss, and A. G. Frantz. 1979. Vasopressin release due to manual restraint in the rat: Role of body compression and comparison with other stressful stimuli. *Endocrinology* 104(3):641-644.

Jahn, G. A. and R. P. Deis. 1986. Stress-induced prolactin release in female, male and androgenized rats: Influence of progesterone treatment. *J Endocrinol* 110(3):423-428.

Kant, G. J., R. H. Lenox, B. N. Bunnell, E. H. Mougey, L. L. Pennington, and J. L. Meyerhoff. 1983. Comparison of stress response in male and female rats: Pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. *Psychoneuroendocrinology* 8(4):421-428.

Khorram, O., J. C. Bedran de Castro, and S. M. McCann. 1985. Stress-induced secretion of alpha-melanocyte-stimulating hormone and its physiological role in modulating the secretion of prolactin and luteinizing hormone in the female rat. *Endocrinology* 117(6):2483-2489.

Krulich, L., E. Hefco, P. Illner, and C. B. Read. 1974. The effects of acute stress on the secretion of LH, FSH, prolactin and GH in the normal male rat, with comments on their statistical evaluation. *Neuroendocrinology* 16(5-6):293-311.

Lang, R. E., J. W. Heil, D. Ganten, K. Hermann, T. Unger, and W. Rascher. 1983. Oxytocin unlike vasopressin is a stress hormone in the rat. *Neuroendocrinology* 37(4):314-316.

Mattheij, J. A. and T. A. van Pijkeren. 1977. Plasma prolactin in undisturbed cannulated male rats; effects of perphenazine, frequent sampling, stress and castration plus oestrone treatment. *Acta Endocrinol* 84(1):51-61.

Morehead, M. H. and R. R. Gala. 1987. Restraint stress depresses prolactin surges in pseudopregnant rats and adrenalectomy does not alter the response. *Life Sci* 41(12):1491-1498.

Morehead, M. H., K. J. Lookingland, and R. R. Gala. 1990. Stress-induced suppression of the prolactin afternoon surge in ovariectomized, estrogen-treated rats and the nocturnal surge in pseudopregnant rats are accompanied by an increase in median eminence dihydroxyphenylacetic acid concentrations. *Neuroendocrinology* 51(2):208-212.

Muir, J. L. and H. P. Pfister. 1986. Corticosterone and prolactin responses to predictable and unpredictable novelty stress in rats. *Physiol Behav* 37(2):285-288.

Neill, J. D. 1970. Effect of "stress" on serum prolactin and luteinizing hormone levels during the estrous cycle of the rat. *Endocrinology* 87(6):1192-1197.

Reynaert, R., S. Marcus, M. De Paepe, and G. Peeters. 1976. Influences of stress, age and sex on serum growth hormone and free fatty acid levels in cattle. *Horm Metab Res* 8(2):109-114.

Riegle, G. D. and J. Meites. 1976a. Effects of aging on LH and prolactin after LHRL L-dopa, methyl-dopa, and stress in male rat. *P Soc Exp Biol Med* 151(3):507-511.

Riegle, G. D. and J. Meites. 1976b. The effect of stress on serum prolactin in the female rat. *P Soc Exp Biol Med* 152(3):441-448.

Seggie, J. A. and G. M. Brown. 1975. Stress response patterns of plasma corticosterone, prolactin, and growth hormone in the rat, following handling or exposure to novel environment. *Can J Physiol Pharmacol* 53(4):629-637.

Siegel, R. A., N. Conforti, and I. Chowers. 1980. Neural pathways mediating the prolactin secretory response to acute neurogenic stress in the male rat. *Brain Res* 198(1):43-53.

Simms, D. D., L. V. Swanson, and R. Bogart. 1978. Effect of collection stress on serum growth hormone levels in pygmy goats. *J Anim Sci* 46(2):458-462.

Yelvington, D. B., G. K. Weiss, and A. Ratner. 1984. Effect of corticosterone on the prolactin response to psychological and physical stress in rats. *Life Sci* 35(16):1705-1711.

Page 39 Moreover, their usefulness is subjected to the same limitations as discussed above, although chronic indwelling vascular catheters and automated blood collection systems may circumvent this limitation to some degree (Abelson et al. 2005).

Atkinson, H. C., S. A. Wood, Y. M. Kershaw, E. Bate, and S. L. Lightman. 2006. Diurnal variation in the responsiveness of the hypothalamic-pituitary-adrenal axis of the male rat to noise stress. *J Neuroendocrinol* 18(7):526-533.

Lightman, S. L., R. J. Windle, M. D. Julian, M. S. Harbuz, N. Shanks, S. A. Wood, Y. M. Kershaw, and C. D. Ingram. 2000. Significance of pulsatility in the HPA axis. *Novar Fnd Symp* 227:244-257.

Rogers, W. R., J. H. Lucas, B. C. Mikiten, H. D. Smith, and J. L. Orr. 1995. Chronically indwelling venous cannula and automatic blood sampling system for use with nonhuman primates exposed to 60 Hz electric and magnetic fields. *Bioelectromagnetics* Suppl 3:103-110.

Windle, R. J., S. A. Wood, N. Shanks, P. Perks, G. L. Conde, A. P. da Costa, C. D. Ingram, and S. L. Lightman. 1997. Endocrine and behavioural responses to noise stress: Comparison of virgin and lactating female rats during non-disrupted maternal activity. *J Neuroendocrinol* 9(6):407-414.

Windle, R. J., S. A. Wood, S. L. Lightman, and C. D. Ingram. 1998a. The pulsatile characteristics of hypothalamo-pituitary-adrenal activity in female Lewis and Fischer 344 rats and its relationship to differential stress responses. *Endocrinology* 139(10):4044-4052.

Windle, R. J., S. A. Wood, N. Shanks, S. L. Lightman, and C. D. Ingram. 1998b. Ultradian rhythm of basal corticosterone release in the female rat: Dynamic interaction with the response to acute stress. *Endocrinology* 139(2):443-450.

Page 39 Many different types of stressors cause the rapid activation of the sympathetic division of the autonomic nervous system (ANS) (Blanc et al. 1991).

Blanc, J., M. L. Grichois, M. Vincent, and J. L. Elghozi. 1994. Spectral analysis of blood pressure and heart rate variability in response to stress from air-jet in the Lyon rat. *J Auton Pharmacol* 14(1):37-48.

Inagaki, H., M. Kuwahara, and H. Tsubone. 2004. Effects of psychological stress on autonomic control of heart in rats. *Exp Anim* 53(4):373-378.

McDougall, S. J., J. R. Paull, R. E. Widdop, and A. J. Lawrence. 2000. Restraint stress: Differential cardiovascular responses in Wistar-Kyoto and spontaneously hypertensive rats. *Hypertension* 35:126-129.

Saunders, P. R., P. Miceli, B. A. Vallance, L. Wang, S. Pinto, G. Tougas, M. Kamath, and K. Jacobson. 2006. Noradrenergic and cholinergic neural pathways mediate stress-induced reactivation of colitis in the rat. *Autonom Neurosci* 124(1-2):56-68.

Stiedl, O., M. Meyer, T. Kishimoto, M. G. Rosenfeld, and J. Spiess. 2003. Stress-mediated heart rate dynamics after deletion of the gene encoding corticotropin-releasing factor receptor 2. *Eur J Neurosci* 17(10):2231-2235.

Tornatzky, W. and K. A. Miczek. 1994. Behavioral and autonomic responses to intermittent social stress: Differential protection by clonidine and metoprolol. *Psychopharmacology* 116(2):346-356.

van den Buuse, M., G. Lambert, M. Fluttert, and N. Eikelis. 2001a. Cardiovascular and behavioural responses to psychological stress in spontaneously hypertensive rats: Effect of treatment with DSP-4. *Behav Brain Res* 119(2):131-142.

Wood, S. K., R. E. Verhoeven, A. Z. Savit, K. C. Rice, P. S. Fischbach, and J. H. Woods. 2006. Facilitation of cardiac vagal activity by CRF-R1 antagonists during swim stress in rats. *Neuropsychopharmacology* 31(12):2580-2590.

Xie, Y. F., Q. Jiao, S. Guo, F. Z. Wang, J. M. Cao, and Z. G. Zhang. 2005. Role of parasympathetic overactivity in water immersion stress-induced gastric mucosal lesion in rat. *J Appl Physiol* 99(6):2416-2422.

Zhang, W. and P. Thoren. 1998. Hyper-responsiveness of adrenal sympathetic nerve activity in spontaneously hypertensive rats to ganglionic blockade, mental stress and neuronglucopenia. *Pflugers Arch* 437(1):56-60.

Page 39 Some stressors may also increase the activity of the parasympathetic division affecting both core body temperature and the gastrointestinal system (e.g., disturbed intestinal absorption, gastric ulceration, colitis; Johnson et al. 2002).

Ray, A., R. M. Sullivan, and P. G. Henke. 1987. Adrenergic modulation of gastric stress pathology in rats: A cholinergic link. *J Auton Nerv Syst* 20(3):265-268.

Saunders, P. R., N. Hanssen, and M. H. Perdue. 1997. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar-Kyoto rats. *Am J Physiol* 273: G486-G490.

Saunders, P. R., P. Miceli, B. A. Vallance, L. Wang, S. Pinto, G. Tougas, M. Kamath, and K. Jacobson. 2006. Noradrenergic and cholinergic neural pathways mediate stress-induced reactivation of colitis in the rat. *Autonom Neurosci* 124(1-2):56-68.

Xie, Y. F., Q. Jiao, S. Guo, F. Z. Wang, J. M. Cao, and Z. G. Zhang. 2005. Role of parasympathetic overactivity in water immersion stress-induced gastric mucosal lesion in rat. *J Appl Physiol* 99(6):2416-2422.

Page 39 For example, telemetry in conscious, unrestrained animals is an effective method for the continuous monitoring of physiologic alterations in heart rate, respiration, blood pressure, ECG, and body temperature (Akutsu et al. 2002).

Azar, T., J. Sharp, and D. Lawson. 2005. Stress-like cardiovascular responses to common procedures in male versus female spontaneously hypertensive rats. *Contemp Top Lab Anim* 44(3):25-30.

Brockway, B. P., P. A. Mills, and S. H. Azar. 1991. A new method for continuous chronic measurement and recording of blood pressure, heart rate and activity in the rat via radiotelemetry. *Clin Exp Hypertens A* 13(5):885-895.

Caplea, A., D. Seachrist, G. Dunphy, and D. Ely. 2000. SHR Y chromosome enhances the nocturnal blood pressure in socially interacting rats. *Am J Physiol Heart Circ Physiol* 279(1):H58-H66.

Harkin, A., T. J. Connor, J. M. O'Donnell, and J. P. Kelly. 2002. Physiological and behavioral responses to stress: What does a rat find stressful? *Lab Anim* 31(4):42-50.

Irvine, R. J., J. White, and R. Chan. 1997. The influence of restraint on blood pressure in the rat. *J Pharmacol Toxicol* 38(3):157-162.

Kramer, K., J. A. Grimbergen, L. van der Gracht, D. J. van Iperen, R. J. Jonker, and A. Bast. 1995. The use of telemetry to record electrocardiogram and heart rate in freely swimming rats. *Method Find Exp Clin* 17(2):107-112.

Lawson, D. M., M. Churchill, and P. C. Churchill. 2000. The effects of housing enrichment on cardiovascular parameters in spontaneously hypertensive rats. *Contemp Top Lab Anim* 39(1):9-13.

Lemaire, V. and P. Mormede. 1995. Telemetered recording of blood pressure and heart rate in different strains of rats during chronic social stress. *Physiol Behav* 58(6):1181-1188.

Nakashima, T., M. Akamatsu, A. Hatanaka, and T. Kiyohara. 2004. Attenuation of stress-induced elevations in plasma ACTH level and body temperature in rats by green odor. *Physiol Behav* 80(4):481-488.

Rubini, R., A. Porta, G. Baselli, S. Cerutti, and M. Paro. 1993. Power spectrum analysis of cardiovascular variability monitored by telemetry in conscious unrestrained rats. *J Auton Nerv Syst* 45(3):181-190.

Sato, K., F. Chatani, and S. Sato. 1995. Circadian and short-term variabilities in blood pressure and heart rate measured by telemetry in rabbits and rats. *J Autonom Nerv Syst* 54(3):235-246.

Schierok, H., M. Markert, M. Pairet, and B. Guth. 2000. Continuous assessment of multiple vital physiological functions in conscious freely moving rats using telemetry and a plethysmography system. *J Pharmacol Toxicol* 43(3):211-217.

Sharp, J., T. Zammit, T. Azar, and D. Lawson. 2002a. Does witnessing experimental procedures produce stress in male rats? *Contemp Top Lab Anim* 41(5):8-12.

Sharp, J. L., T. Zammit, T. Azar, and D. M. Lawson. 2002b. Stress-like responses to common procedures in male rats housed alone or with other rats. *Contemp Top Lab Anim* 41(4):8-14.

Sharp, J. L., T. Zammit, and D. Lawson. 2002c. Stress-like responses to common procedures in rats: Effect of the estrous cycle. *Contemp Top Lab Anim* 41:15-22.

Sharp, J., T. Zammit, T. Azar, and D. Lawson. 2003a. Are "by-stander" female Sprague-Dawley rats affected by experimental procedures? *Contemp Top Lab Anim* 42(1):19-27.

Sharp, J., T. Zammit, T. Azar, and D. Lawson. 2003b. Stress-like responses to common procedures in individually and group-housed female rats. *Contemp Top Lab Anim* 42(1):9-18.

Sharp, J., T. Azar, and D. Lawson. 2005a. Effects of a cage enrichment program on heart rate, blood pressure, and activity of male Sprague-Dawley and spontaneously hypertensive rats monitored by radiotelemetry. *Contemp Top Lab Anim* 44(2):32-40.

Sharp, J., T. Azar, and D. Lawson. 2005b. Selective adaptation of male rats to repeated social encounters and experimental manipulations. *Contemp Top Lab Anim* 44(2):28-31.

van den Buuse, M. 1994. Circadian rhythms of blood pressure, heart rate, and locomotor activity in spontaneously hypertensive rats as measured with radio-telemetry. *Physiol Behav* 55(4):783-787.

van den Buuse. M., S. A. Van Acker, M. Fluttert, and E. R. De Kloet. 2001b. Blood pressure, heart rate, and behavioral responses to psychological "novelty" stress in freely moving rats. *Psychophysiology* 38(3):490-499.

Wood, S. K., R. E. Verhoeven, A. Z. Savit, K. C. Rice, P. S. Fischbach, and J. H. Woods. 2006. Facilitation of cardiac vagal activity by CRF-R1 antagonists during swim stress in rats. *Neuropsychopharmacology* 31(12):2580-2590.

4

Avoiding, Minimizing, and Alleviating Distress

The simplest approach to avoiding, minimizing, and alleviating distress in laboratory animal care and use is to follow the principles of the *Three Rs*—refinement, reduction, and replacement. It is important, however, to strike a balance between the integrity of research outcomes and the welfare of animals used. Investigators, veterinarians, and animal care personnel should function as a team and base their decisions on professional judgment, best practices, and thorough clinical evaluation of distressed animals. Refining aspects of housing, husbandry, enrichment, and socialization helps alleviate or prevent distress. Refining the experimental design, utilizing humane or surrogate endpoints, and using the appropriate statistical analyses (including an accurate calculation of sample size) helps reduce the numbers of animals used and alleviate some of their distress. A team approach is necessary to address treatment options for distressed animals. When using procedures that intentionally result in distress, the investigator should, in consultation with the veterinarian and the IACUC, develop a plan that will establish limits to the levels of distress allowed.

INTRODUCTION

Established ethical, regulatory, and scientific practices, standards, and policies mandate avoiding animal distress whenever possible. However, if research or regulatory testing objectives cause a research animal to experience distress, it is incumbent upon the animal user to identify the cause(s) of distress, attempt to minimize its duration and intensity, and/or provide the

means for the animal to cope. The simplest approach is to follow the principles developed by Russell and Burch known as the *Three Rs* (Russell and Burch 1959). The *Three Rs* are (1) **refinement** of the protocol to minimize or eradicate distress for the species used (e.g., by employing nonclinical [e.g., molecular measurements] or defined [e.g., tumor growth instead of survival] endpoints; giving positive rewards; changing or refining the data/sample collection methods; or instituting species-specific husbandry refinements such as enrichment); 2) **reduction** of the number of animals used to the absolute minimum necessary (based on appropriate statistical sample size determination or other field-specific methods), particularly if they are likely to experience unavoidable distress; and (3) **replacement** of an animal with a nonanimal model or a less sentient species, usually of a lower phylogenetic order, such as a primitive invertebrate.

As discussed in Chapters 2 and 3, distress may result from a single intense or prolonged stressful experience or from several simultaneous stressors that might individually cause stress but not likely distress. Therefore, mitigating some potentially stressful circumstances, such as husbandry schedules, may allow an animal to better adapt to other stressors such as experimental procedures. It is important to weigh any possibly adverse impact of replacement, refinement, and reduction on scientific outcome against both the negative impact of failure to avoid, minimize, or alleviate stress and distress on the research data and the numbers of potentially wasted animal lives.

This chapter identifies approaches to avoid or minimize distress through the alleviation and minimization of stress, in both the care *and* use of laboratory animals. The chapter also suggests ways to alleviate distress that cannot be avoided or minimized because of scientifically justified research protocols, and addresses the challenges and compromises that arise when the object of research itself is the study of stress and distress. This chapter is not intended to be comprehensive but to provide investigators and IACUC members with an awareness of common problems and useful strategies. The Committee encourages all who are involved in laboratory animal care and use to think creatively when considering solutions to specific circumstances.

AVOIDING OR MINIMIZING DISTRESS IN LABORATORY ANIMAL CARE

Most animals are able to cope with a relatively wide range of naturally occurring environments, but such environments are not usually characteristic of laboratory animal facilities. Research environments are generally designed as a compromise between the needs of the animal, the user, and

the husbandry staff. While most animals continue to function normally[1] in animal care facilities, some do not adapt successfully to a laboratory environment (see Chapter 3) and it is important to consider the effects of their maladaptation on both the research and the animal's welfare. The Committee notes the lack of consensus among the scientific community as to the impact and quantification of the effects of the major stressors on an animal's welfare and the most appropriate modifications in response to these perturbations. The Committee also notes that relevant research data to answer many of the issues undertaken in this chapter are still inadequate and often absent. Therefore the text below is a combination of available scientific literature and professional judgment and expertise.

Housing

Potential environmental stressors that may lead to stress and distress include levels of ambient light, noise, vibrations, fluctuations or extremes in temperature, husbandry practices, and facility maintenance (e.g., construction, vibration). The degree to which these stressors can lead to distress is highly variable; in many cases, signs of distress appear only after prolonged stimulation, as noted in Chapters 2 and 3. Most laboratory rodents, for example, are nocturnal but their housing environment may make it difficult for them to withdraw from brightly lit areas, they may be handled during their somnolent period, or they may be exposed to sudden or loud noises. It has been demonstrated that exposing rodents (normal, albino, or transgenic) to excessively bright or continuously high levels of light can cause permanent damage such as retinal injury (Kaldi et al. 2003; Wasowicz et al. 2002). In these cases it is appropriate to reduce the levels of light (for more information see pages 34-35 of *The Guide for the Care and Use of Laboratory Animals* [NRC 1996]) and to provide refuges that enable the animals to hide from it. Similarly, just as noise can be a stressor and affect the health of humans (Passchier-Vermeer and Passchier 2000; Tomei et al. 2000), sound and ultrasound can be stressful and cause external variation

[1]In the interest of uniformity and adherence to commonly accepted normative values, the Committee chose the word "normal" to describe the life of a laboratory animal that, if not subjected to experimental procedures, would live mostly undisturbed. Implicit in this position is the notion that many laboratory animals are "artificial constructs" for which the question of whether they could successfully adapt or live in the natural world is a philosophical exercise. The Committee, however, recognizes the argument put forth over the last 15 years that any artificial environment is abnormal, because it does not allow the laboratory animal to function according to its natural predisposition, and as such, behavioral variations among laboratory animals are only degrees of abnormality (Garner 2005, 2006; Würbel 2000). However, the definition of "natural predisposition" is not entirely clear when the subject matter is laboratory animals, especially those that have been domesticated over many generations.

in animal data (Clough 1982; Milligan et al. 1993; Sales et al. 1999; Shoji et al. 1975). Noise in dog kennels can reach levels that can damage human hearing, and that may well have an impact on dog hearing and physiology as well (Hubrecht et al. 1997; Sales et al. 1997). Vibration has been demonstrated to be a stressor in both poultry (Abeyesinghe et al. 2001) and pigs (Perremans et al. 1998); investigators should, therefore, strive to minimize common sources of vibration such as ventilation machinery in adjacent rooms or on cage racks (Clark 1997). In addition to such continuous background disturbances, the effects of isolated environmental insults may also cause distress in some species and models.

For experimental and comfort reasons it is best to maintain animals in their thermoneutral zone (NRC 2006, pages 39-45). The Committee notes the discrepancy between the temperature recommendations for housing rodents in the *Guide for the Care and Use of Laboratory Animals* (NRC 1996, page 32) and those put forth in the *Guidelines for the Humane Transportation of Research Animals* (NRC 2006, pages 39-45) due to new evidence used in the later publication. Rabbits are very susceptible to heat stress (Marai et al. 2002). Singly housed mice prefer ambient temperatures of 28-30°C while group-housed mice prefer a slightly colder environment with ambient temperatures of 24-27°C (NRC 2003b, page 97). The provision of nesting materials, refuges, or nest boxes for rodents, or areas within an enclosure with different levels of heating or cooling (i.e., heated areas for dogs) allows the animals to control their microclimate. Moreover, long-term housing in cages with mesh floors where adequate bedding or nesting materials cannot be provided can also result in stress, distress, or more obvious deleterious effects, such as foot ulcerations and arthritis.

Enrichment

Barren environments may not meet the species-specific needs of an animal. In addition to their impact on welfare these conditions can adversely affect the validity of experimental data (Sherwin 2004). Such environments can cause distress as shown by the development of abnormal behaviors (see Chapter 3) or by experiments in which animals, when given the choice to self-medicate with anxiolytics, consume larger proportions of midazolam-water solution than their littermates housed in enriched environments (Sherwin and Olsson 2004). In contrast, supporting evidence has shown that biologically relevant enrichment can help avoid the development of abnormal behaviors (see Chapter 3), although it may not alleviate previously established patterns. Moreover, as Olsson and Dahlborn have shown, some animals exhibit clear preferences and will work to access these enrichments (Olsson and Dahlborn 2002). In mice, environmental

enrichment may attenuate anxiety and stress and restore immune response (Benaroya-Milshtein et al. 2004). It can also slow disease progression, a consequence that in some circumstances might interfere with the research aims but that may also provide insights into new or better treatments or new research avenues (Hockly et al. 2002). Enrichment can thus improve welfare, reduce stress, and improve the quality of data obtained from the animals in situations where such enrichment does not compromise the anticipated research outcomes. However, the effect of environmental enrichment on stress responses can vary depending on species or strain, the type of enrichment used, the stressor employed, and the type(s) of stress response(s) evaluated (Bardo et al. 2001; Belz et al. 2003; Green et al. 2002; Lawson et al. 2000; Marashi et al. 2003; Moncek et al. 2004; Roy et al. 2001; Schrijver et al. 2002; Sharp et al. 2005; Tsai et al. 2002).

Ideally, enrichment devices or strategies should draw on previous literature or research that shows that they are beneficial to the animals and have no unexpected adverse effects on their health, and that their use does not jeopardize experimental outcomes and research goals through the introduction of uncontrolled variables, increased variability, and/or inter-experimental variance leading to a need for more animal studies (Baumans 2005; Bayne 2005; FELASA Working Group Standardization of Enrichment 2006). Benefiel and colleagues suggest the need for evidence-based evaluation of "mandatory" enrichment practices for all laboratory animal species (Benefiel et al. 2005). Meier and colleagues have shown that enrichment (in the form of various housing supplements) can increase the acute stress response (as evidenced by elevated heart rate and body temperature) of individually housed mice (Meier et al. 2007). Recent evaluation of the effect of enriched environment on genetically engineered fibulin-4 knockout mice (fibulin-$4^{+/-}$) has shown that knockouts in enriched cages had fewer disorganized regions on their arterial walls than knockout littermates housed in standard cages. These results suggest that the type of housing environment may interfere with the expected phenotype of genetic manipulations and with the experimental outcomes (Cudilo et al. 2007). However, despite a lack of adequate pilot studies, background data, or published information, even such highly controlled conditions as toxicology studies have effectively adopted appropriate enrichment enhancements (Dean 1999; Turner et al. 2003). Faced with the absence of unequivocal scientific evidence for data-driven enrichment standards and aware of the potential for unexpected consequences by the indiscriminate use of enrichment strategies, the Committee makes its recommendations guided by best practices and expert professional judgment in an attempt to balance the need to safeguard animal welfare while maintaining scientific excellence.

Socialization

It is generally appropriate to house naturally gregarious animals in compatible social groups unless there are scientific or welfare reasons not to do so (National Health and Medical Research Council 2004; Canadian Council on Animal Care 1993; Council of Europe 2006; NRC 1996). Social housing can activate stress responses involving the HPA axis in rats, but when a wider range of measures is taken into account, overall, social housing is neither stressful nor harmful (Hurst et al. 1997, 1998). For example, even macaques fitted with a cranial implant could be paired with another compatible macaque without it inflicting damage to the device or interfering with the research goals (Roberts and Platt 2005). Furthermore, a considerable body of evidence indicates that housing naturally sociable animals (e.g., rats, mice, dogs, primates) in solitary conditions can result in stress and harm (Baker 1996; Eaton et al. 1994; Hetts 1991; Hubrecht 1995; Novak 2003; Patterson-Kane 2002; Sharp and Lawson 2003; Van Loo et al. 2004). Even cats, which are not particularly gregarious, can benefit from social housing (Council of Europe 2006). It is therefore important to provide thorough scientific rationale for solitary housing.

Disruption of established social groups, pairing (for additional information see Appendix), or the introduction of animals to larger preformed units are all potential causes of aggression or stress. As a husbandry refinement, therefore, social groups should be established early, and disruption of established groups should be minimal, as demonstrated in studies of mice, rabbits, and cats (Bradshaw and Hall 1999; Jennings et al. 1998; Morton et al. 1993; Sharp et al. 2002b). Close cooperation with the supplier or breeder may be necessary to promote group formation and ensure minimal disruption of group dynamics. Adequate socialization to both humans and conspecifics at an early age may also help prevent subsequent stress and distress (Council of Europe 2006).

Animals housed in social groups generally need adequate space as well as objects in their enclosure to allow them to modulate their social interactions. However, some structures can actually trigger aggression, as shown in certain strains of male mice (Haemisch and Gartner 1994). Because competition for resources often triggers aggression, the provision of sufficient or separate feeding devices for some species (e.g., dogs, cats, pigs) can help minimize the risk of fights during feeding. For other species, such as mice and marmosets, that regulate social interactions through olfactory markings, appropriate cage changing and cleaning routines can minimize social disruption. For example, decreasing the frequency of cage cleaning or leaving some older bedding can help maintain tolerance between familiar male mice (Hurst et al. 1993) and transferring nesting material between cages can positively influence several stress-related physiological

parameters (Van Loo et al. 2004), while retaining scents in certain areas of the cage (e.g., the top grill) may increase aggression (Gray and Hurst 1995).

Housing animals in groups that are not compatible (e.g., certain strains of postpubertal male mice) can result in aggression, stress, distress, injuries, and even death. While all social groups should be monitored for compatibility, this is particularly important immediately after the formation of the group. Animals that require individual housing may benefit from visual, auditory, olfactory, and even tactile contact with other animals, as such interactions are thought to improve the welfare of all animals involved.

Husbandry

While predictable variations in housing conditions can be a useful component of enrichment, unpredictability in animal care can be stressful and potentially distressing if prolonged or extreme. Even routine cage cleaning and changing can be stressful or become distressful if not consistently and routinely performed in a gentle manner (for more details see Chapter 3). Cardiovascular and behavioral changes, such as elevated blood pressure, heart rate, and movement, lasted up to 60 min after changing the cages of adult male Sprague Dawley rats (Duke et al. 2001). Cage and room cleaning also disrupt olfactory environments that are important to animals that depend on their sense of smell to socialize (Gray and Hurst 1995).

Because husbandry procedures can be stressful to the laboratory animal, performing more than one simultaneously (e.g., weighing animals at the time of transfer to clean cages) may decrease the handling stressor in some species if such arrangements are possible. Alternatively, more frequent, gentle, predictable handling may habituate an animal and thus minimize handling stress. In species such as dogs and primates, strategies such as positive rewards and operant conditioning techniques can minimize stress and thus the potential for distress for both animals and handlers (Prescott and Buchanan-Smith 2003; Weed and Raber 2005). Many techniques that minimize stress in husbandry—such as combining husbandry handling with habituation and handling for research purposes, acclimation to new environments, positive reinforcements, operant conditioning, and well-trained staff—can be helpful tools for the overall reduction of stress and distress; for further information see *ILAR Journal* 47(4).

It is extremely important to involve both research personnel who are knowledgeable and skilled in current methods and well-trained and attentive animal care employees. Individuals who understand the normal behavior and appearance of animals and have mastered the appropriate handling and restraint techniques are quick to identify abnormal clinical signs that may be indicators of distress. Rapid identification and prompt attention to

the stressors facilitates avoidance, minimization, and alleviation of distress, if such interference is not incompatible with the objectives of the research protocol.

AVOIDING OR MINIMIZING DISTRESS IN LABORATORY ANIMAL USE

Refining the Experimental Design

A variety of strategies to refine the research protocol can help minimize animal stress and distress. A thorough literature review is vital for a critical analysis of the suitability, applicability, and validation of the proposed methodology. This section addresses the importance of correct statistical methods on the number of animals used. Choosing an earlier stage for an intervention (mild severity) or employing a different approach to arrive at the same research objective might work as effectively as waiting for later impacts of high severity and substantial distress. Examples of less stressful approaches include not allowing a tumor to grow to the point that it affects mobility before starting an experimental treatment, replacing long fasts as a motivating factor with the work-by-reward method, selecting a smaller stimulus to elicit a response before high-intensity stimuli are employed for the evaluation of a novel analgesic, and keeping the withdrawal of food and water in learning experiments to the minimum time necessary (Morton 1998; Morton and Hau 2002; NRC 2003a).

If a potential source of distress is the data-gathering or sample collection process itself, a less invasive method may be appropriate. For example, if the experimental design justifies it, the one-time surgical implantation of vascular lines and sensors can replace manual restraint for frequent blood collection or other physiological measurements, to avoid repeatedly subjecting the animal to stressful experiences (proper aseptic techniques and frequent peridermal maintenance is required when handling such surgical implants; for more information see chronically instrumented nonhuman primates in Broadbear et al. 2004). This is a common strategy for animals in chronic studies. However, it may be necessary to strike a balance if repeated surgery is necessary in order to replace batteries or sensors (Hawkins et al. 2004; Morton et al. 2003). Obviously, the constraints of the study will determine the appropriateness of alternative techniques, which may not be suitable for some types of studies or housing systems (Vahl et al. 2005).

Further examples of less stressful options (more information on the severity of stress caused by these methods is included in Chapter 3) include the use of oral or rectal swabs, plucked hair, or tissue from ear punches in place of tail tip amputation for the purpose of genotyping (Hawkins et al. 2006; Pinkert 2003; Robinson et al. 2003), and the measurement of cortisol

and other steroids in samples from saliva (Aardal and Holm 1995; Kiess et al. 1995) and plucked hair (Davenport et al. 2006). Even less handling is involved when samples are taken from voided urine, feces, expired air, and shed hair (Poon and Chu 1999), if these methods are validated for the species under study. Other noninvasive techniques for data collection include sound recordings (Holy and Guo 2005), cameras (Hobbs et al. 1997), or noninvasive, sensor-laden apparel simply worn by the animal (Jarrell et al. 2005).

Humane Endpoints

Validated endpoints that occur earlier in the course of the protocol and involve no detectable indication of disease, injury, or abnormal behavior can prevent or minimize distress in experimentation and testing. The use of humane endpoints (i.e., "end a study earlier to avoid or terminate unrelieved pain and/or distress"; Stokes 2000) or surrogate endpoints (i.e., those that can reliably substitute for more distressing or painful phenomena) is especially applicable in scientific disciplines that focus primarily on molecular and cellular phenomena associated with disease (Morton 2000; Hendriksen and Morton 1999). In these cases, biochemical changes may be detectable at early stages in the disease process, prior to the manifestation of clinical signs consistent with distress. For example, elevated white blood cell counts are detectable in leukemia models before illness becomes obvious and serum biochemical values often change in early stages of toxicity before animals appear ill (Poon and Chu 1999). Thus, taking measurements or collecting samples from animals *before* the appearance of any clinical signs (including all clinical manifestations, not only those related to distress) is desirable, especially when the signs themselves are not the study's focus. In such cases, the predictive value of validated endpoints may permit early euthanasia of these animals and postmortem collection of data or samples (for additional information see Appendix). Alternatively, a clinically normal animal could be anesthetized before a distressful procedure and euthanized before regaining consciousness.

Familiarity with certain procedures or experimental protocols often allows for predicting the course of adverse clinical signs and distress. In many instances death results from indirect effects such as dehydration and is not related to the response variable under study. In mice, for example, progressive hypothermia due to low food intake will cause an animal's death over several days. However, distress can be minimized through the use of validated humane endpoints, such as euthanizing the animals at the first recording of low body temperature (Morton 1998; Soothill et al. 1992). The choice and use of endpoints should be part of the experimental protocol whenever possible.

The Value of Statistics

Pilot Studies

For certain experimental procedures (e.g., acute toxicity protocols), the scientific literature or the complexity of the biological system under study suggests that distress is possible but not predictable. Distress may also result from investigator inexperience, the use of technically demanding procedures, or the establishment of a new animal model. In those cases, a pilot study with fewer animals may be appropriate in order to establish proof-of-concept or to achieve a learning curve before seeking approval for the use of more animals. Other benefits of pilot studies include the collection of useful preliminary data to better estimate the appropriate sample size, the identification of unanticipated adverse effects, and opportunities for refinements (e.g., endpoint determination and monitoring schedules). Pilot studies, however, are not appropriate for all protocols, as they can also lead to an increase in the number of animals needed or the unnecessary consumption of valuable reagents and other limited resources.

Sample Size Determination

Appropriate statistical analyses are useful for the reduction of the numbers of animals used and determination of the desired statistical power and minimum sample size values (*n*) needed to discriminate between significantly different groups or endpoints (NRC 2003a). Several publications reviewing the use of animals in experimental protocols found that the majority of studies evaluated did not have adequate statistical power to detect even a large difference between experimental groups (Chung et al. 2002; Dirnagl 2006; Gold et al. 2005; Riley et al. 1998). In the preferred method of sample size determination, the "power analysis", the experiment should be designed so that there is at least an 80 percent probability (i.e., a minimal statistical power of 0.8) of detecting a difference ("the effect size") of a specific magnitude between experimental groups. According to Shaw and colleagues, the "effect size is the magnitude of the difference between treatment and control means, which the experiment is to be designed to detect" (Shaw et al. 2002). An adequate sample size determination is necessary to ensure that the effect size achieves both scientific validity and statistical significance.

It is crucial for researchers to perform the sample size calculation before initiating a study in order to reduce the number of animals utilized *and* ensure that the number of animals (sample size, *n*) will provide scientifically valid data. This calculation derives the sample size necessary to detect a statistically significant effect at the desired power level. There are four

factors that must be known or estimated to calculate a sample size (Dell et al. 2002):

1. Effect size: the difference between experimental groups (see above);
2. Population standard deviation: the variability within a population;
3. Power level (1-β): the probability that a difference of specific magnitude between groups will be detected (at least 80 percent); and
4. Significance level (α): the probability that a difference between groups is due to chance alone (classically defined as 0.05 or 5 percent).

Power analysis cannot be applied in all situations. For example, in cases in which experiments measure many variables, it is quite difficult to specify and almost impossible to calculate the effect size for each one. In microarray analyses, where thousands of observations per animal are collected, it is not possible to postulate an effect size for each one. Furthermore, power analysis requires an estimate of the standard deviation of the population, which may not always be available. In these situations, other methods of sample size determination may be more appropriate (Festing et al. 2002; Mead 1988).

After calculating the sample size, researchers should consider additional ways to further reduce it. For example, because the power and significance levels have been set *a priori* (i.e., prior to the sample size determination), increasing the effect size or decreasing the population standard deviation could result in a smaller sample size without sacrificing power. A sample of the various methodologies that have been described includes:

1. Decreasing measurement error (will decrease sample variance and increase sensitivity);
2. Choosing appropriate animal strains (helps control variation; for example, the use of isogenic or inbred murine strains may be more appropriate than outbred ones in some experimental designs (Festing and Altman 2002; Festing et al. 2001);
3. Utilizing endpoints that are continuous rather than dichotomous (continuous data require smaller sample sizes to detect a desired difference between experimental groups);
4. Utilizing the repeated measures experimental design approach (i.e., each animal acts as its own control, decreasing the overall population variability);
5. Decreasing the number of experimental groups (i.e., utilizing the minimum data needed to disprove the null hypothesis; for example, by reducing the number of points of a dose-response curve). This method should be considered in relation to the type of statistical

analysis performed. In a linear regression model the usefulness of collecting data points in the middle of the cluster is debatable, unless a curve is expected;

6. Clustering several experiments around a shared control group to avoid exposing more animals to distress than necessary (e.g., using one control batch against multiple treatment batches; or one control group for multiple doses; Clark 2002; Dell et al. 2002; Sterne and Smith 2001). Historical controls may be useful in toxicological evaluations, safety assessments, and other studies where genetically defined rodent strains are used but no significant genetic drift between generations has been detected. Although their application does reduce the number of animals used, historical controls are not appropriate for all studies and should only be used in the correct scientific context to avoid their substantial limitations.

In the event that treatment and control groups experience different degrees of distress it may be possible to reduce the number of animals subjected to high distress levels and increase those subjected to lower levels in order to maintain the desired level of statistical power. Because this operation will require a greater number of animals than the original calculated minimum (Sedcole 2006), it is advisable to consult a statistician to ensure that statistical power is not compromised. Similarly, the use of appropriate sequential experimental designs can result in a reduction in the numbers of animals that experience distress, as this technique allows the analysis of data as they accumulate (Waterton et al. 2000).

Sufficiently large sample sizes can make even ephemeral differences between groups statistically significant. The Committee emphasizes the need to consider whether a statistically significant difference is actually biologically relevant. Protocols that propose large sample sizes should offer scientifically and statistically valid justification for the high numbers with regard to the biological system or phenomenon studied or the way the data will be used (e.g., in safety testing and the categorization of chemicals).

The Development of New Technologies

The use of minimally invasive imaging technologies is another approach to reducing the number of animals used in experimentation and has already proven beneficial to animal models of cancer. Conventionally, large numbers of mice are inoculated with tumor cells whose progress (e.g., growth, metastasis) prior to euthanasia is usually distressful. Prelabelling tumor cells with fluorescent markers and tracking them over time in each animal with sophisticated imaging equipment is effective and requires fewer animals (Weissleder 2006). Moreover, the use of sequential longitudinal imaging is

a refinement approach that makes it possible to measure tumors so precisely that the animal may be euthanized before any clinical signs arise. This method by itself may greatly reduce the numbers of animals estimated by the sample size determination. Other animal models that benefit from new imaging technologies include those for cardiovascular diseases (labeled cells; Jaffer et al. 2006) and inflammatory bowel diseases (colonoscopy; Becker et al. 2005).

ALLEVIATING DISTRESS IN LABORATORY ANIMALS

As has been noted in Chapter 3, even with reasonable steps to avoid or minimize housing and husbandry-related stressors, distress may still unexpectedly appear once a protocol begins or following a change in husbandry. Many of the steps involved in the alleviation of distress, such as a team management approach and prompt veterinary action, are identical to the procedures described in Chapter 3 for recognizing and assessing the presence of distress. However, before implementing any response plan, the principal investigator/study director and veterinarian or designee should review the objectives of the protocol to determine if the alleviation of distress would adversely affect the research project. Identification of a refinement after approval of a protocol should include amendment of the protocol to adopt this change. If the distress is anticipated or results from a significant husbandry error, regulations require notification of the Institutional Animal Care and Use Committee (IACUC) and possibly of regulatory agencies as well, especially if animal distress results in protocol suspension (DHHS 2005; USDA 2005). Table 4-1 provides an algorithm for responding to unexpected animal distress.

Medical conditions unrelated to the study objectives (e.g., spontaneous self-injurious behavior, fight-related injuries, newly diagnosed ectoparasite infestations) may be treatable without compromising the study. However, additional diagnostic tests may be necessary and even, depending on the therapeutic interventions selected, the removal of the animal from the study, either temporarily or permanently. In addition to eliminating the underlying cause, treatment modalities to address the behavioral signs may include changing the environmental parameters (such as cagemate, caging type, or housing location [Fontenot et al. 2006]; administering analgesics or anxiolytics; engaging in behavior modification and training [Reinhardt 2003; Schapiro et al. 2001]); providing environmental enrichment; dispensing psychotropic medications; or, in severe cases, euthanizing the animal. In one case, environmental enrichment decreased abnormal behaviors in pigtail macaques that could not be socially housed (Kessel and Brent 1998). In contrast, the presence of puzzle feeders, which encouraged manipulation, did not reduce self-injurious behaviors in rhesus monkeys (Novak et

TABLE 4-1 Example of a decision and response algorithm for unanticipated distress in laboratory animals

Animal Issues	Program Issues
▸ Promptly communicate initial observations to principal investigator/study director, clinical veterinarian, facility manager.	• Promptly and accurately document clinical signs and treatments in the animal's record.
▸ Assess animal's clinical status and treatment options with respect to the protocol.	• Evaluate other animals on the same protocol or housed nearby to determine if more animals are possibly in similar distress.
▸ Administer emergency veterinary care if indicated and after consultation with the principal investigator/study director.	• Determine if the distress and accompanying clinical signs are a consequence of experimentation, husbandry error, or other cause.
▸ If the animal's condition is grave and the principal investigator/study director (or designee) cannot be contacted, the animal may be euthanized at the direction of the clinical veterinarian.	• Reduce or eliminate the source(s) of distress, if known and if compatible with the aims of the protocol.
▸ Institute precautionary measures and supportive care if indicated.	• Notify the IACUC (and possibly regulatory agencies) of significant animal distress.
	• Amend the protocol to avoid or reduce distress in more animals. If altering the protocol will compromise scientific aims or regulatory endpoints, assign animals to a more severe pain/distress category.

al. 1998), while Coleman and colleagues demonstrated that the ability of individual monkeys to respond to conventional training methods is closely correlated with their unique temperament (exploratory or inhibited personalities; Coleman et al. 2005).

The actual causes of distress may also lead to sequelae that require attention even if the underlying cause is not treatable. For example, the clinical signs of a distressed animal often include dehydration and weight loss resulting from anorexia. Provision of supplemental fluids and nutrition may relieve the compounding impact of dehydration or poor body condition on the compromised animal. Supplemental heat, cooling, bedding, social housing, and human companionship are other strategies that make a

distressed animal more comfortable. Regardless of the approach selected, it is essential to maintain the dialogue between the investigator, veterinarian, and animal care personnel throughout the treatment phase, because the prognosis and the status of the animal's condition may change.

Distress resulting from behavioral problems resistant to the relatively simple and straightforward approaches listed above can be especially difficult to treat. It may be appropriate to consider psychotropic medications such as anxiolytics, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and neuroleptics if they are compatible with the research protocol. SSRIs and TCAs have been effective in the treatment of animals with repetitive, self-injurious, and anxiety-based behaviors. A firm diagnosis will aid in the choice of medication, as these drugs have been used to treat assorted behavioral problems in multiple species with varying levels of success (for studies on monkeys see Fontenot et al. 2005; Tiefenbacher et al. 2003, 2005; Weld et al. 1998). Taylor and colleagues used a combination of chlorpromazine, buprenorphine, and environmental enrichment to successfully treat a self-injurious behavior in a rhesus monkey (Taylor et al. 2005). Hugo and colleagues showed that fluoxetine had some efficacy in the reduction of stereotypies in captive vervet monkeys (Hugo et al. 2003). Recent studies have shown that stereotypic behavior in mice responded to self-administered anxiolytics (Olsson and Sherwin 2006). Furthermore, opioid antagonists have been used to treat behaviors with a self-rewarding effect in sows (Cronin et al. 1985). An accurate diagnosis and the preparation of a behavior modification plan should precede the initiation of therapy with any psychotropic medications. The Committee notes that, while interest in the use of psychopharmacological treatment for behavioral modifications is growing, limited research data exist relevant to the effects of these drugs on animal behavior. The Committee cautions that there should be appropriate justification for their use (which should not be the first line of defense), that other behavioral modification measures should be implemented, and that these should be accompanied by careful monitoring of the animal.

Decisions to treat, not treat, or euthanize animals with a severe condition or a poor prognosis should involve the entire research and veterinary support team, whose members should make every possible effort to achieve consensus on the decision regarding the fate of the animal. Regulations, however, mandate that the institution's Attending Veterinarian retain the ultimate responsibility and authority over the final disposition of the animal (see Figure 4-1). Decisions that call for euthanasia should follow approved methods, which are regularly updated and published (AVMA 2007). Only skilled, compassionate persons, with properly maintained equipment, should perform euthanasia. Proper handling of animals prior to euthanasia is important to avoid inducing further and unnecessary distress. Sources of

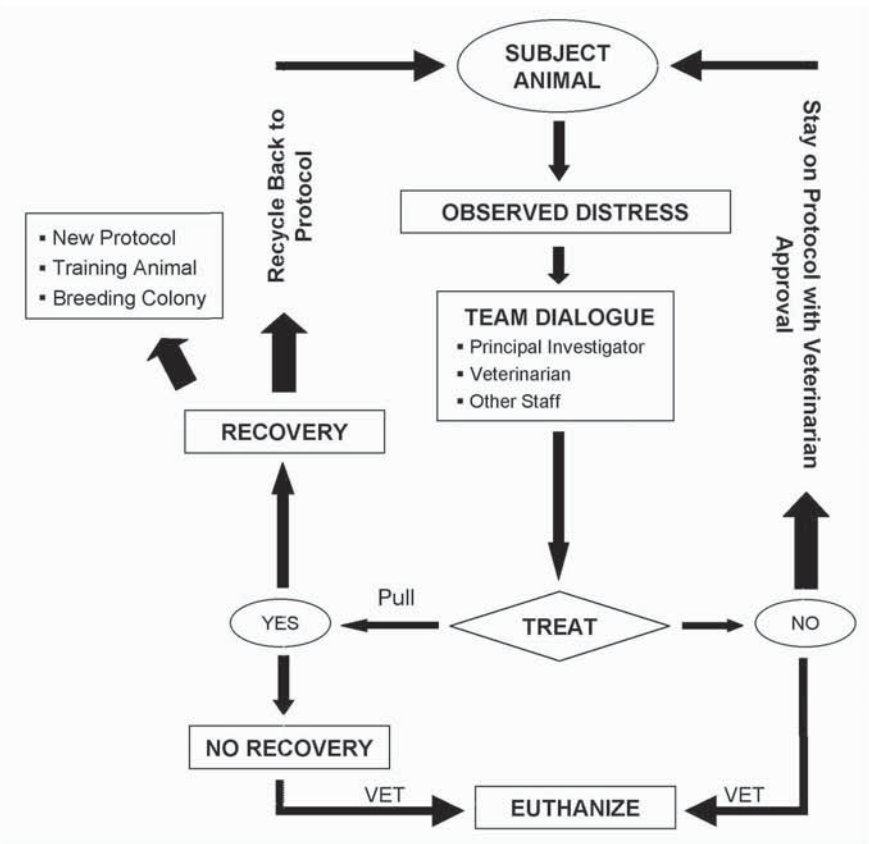


FIGURE 4-1 Distressed animal: Team dialogue on decision making. The decision to not treat an animal would depend on the cause of the distress and the severity of the animal's condition. If the distress is appropriately caused by the research protocol, then the animal will either remain on the study without treatment or—if severely compromised—euthanized. If the distress is caused by an external perturbation, such as husbandry issues, that can be corrected without a direct therapeutic intervention on the animal (which might interfere with protocol), then again the animal would remain in the study without treatment, but the environmental causes would have to be addressed.

distress include, but are not limited to, improper grouping with incompatible conspecifics or other species; lack or withdrawal of food, water, or clean bedding; and inappropriate noise levels and light cycles, particularly if the interval before euthanasia is long. Last, not least, it is essential to ensure that the animals are truly dead before their disposal.

STUDYING DISTRESS

Distress in humans may be more widespread (or at least more readily recognized) than that observed in nonhuman animals because of unique human cognitive capacities, such as the ability to clearly communicate threatening, dangerous, or painful conditions; to remember these circumstances and their consequences over extended periods of time; and to apply the emotions engendered to other stimuli on the basis of verbal or categorical concepts (Sapolsky 1994). A substantial proportion of the American population will at some point suffer from an illness that is distressing or even incapacitating (e.g., depression or a severe anxiety disorder). Many of those afflicted present with no specific experiential basis for their disorder, which suggests that our society's efforts to prevent and/or control intense and chronic stressors, even if relatively successful, may not prevent these maladies.

A significant portion of research with laboratory animals deals with pathology resulting in distress, incapacitation, or death for the animals. While it is often possible to study incapacitating or lethal conditions while using palliative agents or euthanasia in order to alleviate or preclude animal distress, it is not possible to adequately investigate distress itself without allowing it to occur. While it is therefore desirable to reduce distress in laboratory animals, this should not extend to eliminating all of it. Animal models have provided insight into the anatomical and molecular bases of various human distresses (Blanchard and Blanchard 2005; Herman et al. 2005; Maier and Watkins 2005; Phelps and LeDoux 2005). An attempt to totally eliminate the study of distress would imply abandoning the major goal of biomedical research: to understand and find therapeutic solutions for conditions that continue to plague a significant portion of humanity as well as nonhuman animals.

With care and attention, it should be possible to attain the optimum goal of reducing distress even while continuing to investigate it. When using procedures that intentionally result in distress, the investigator, in consultation with the veterinarian and the IACUC, should develop a plan that will establish limits to the levels of distress allowed. Appropriate methods include measures to alleviate distress following completion of the procedures or attainment of the research aims (e.g., maximum allowable weight loss as a percentage of normal body weight). In line with the important goal of extrapolating such research to specific human conditions or disease states, the limits chosen should be sensitive to the goals of the research project and the wider scope of distress-related phenomena to which the project is potentially relevant.

REFERENCES

Aardal, E. and A. C. Holm. 1995. Cortisol in saliva—reference ranges and relation to cortisol in serum. *Eur J Clin Chem Clin* 33(12):927-932.

Abeyesinghe, S. M., C. M. Wathes, C. J. Nicol, and J. M. Randall. 2001. The aversion of broiler chickens to concurrent vibrational and thermal stressors. *Appl Anim Behav Sci* 73(3):199-215.

AVMA (American Veterinary Medical Association). 2007. 2007 AVMA Guidelines on Euthanasia.

Baker, K. C. 1996. Chimpanzees in single cages and small social groups: Effects of housing on behavior. *Contemp Top Lab Anim* 35(3):71-74.

Bardo, M. T., J. E. Klebaur, J. M. Valone, and C. Deaton. 2001. Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. *Psychopharmacology* 155(3):278-284.

Baumans, V. 2005. Environmental enrichment for laboratory rodents and rabbits: Requirements of rodents, rabbits, and research. *ILAR J* 46(2):162-170.

Bayne, K. 2005. Potential for unintended consequences of environmental enrichment for laboratory animals and research results. *ILAR J* 46(2):129-139.

Becker, C., M. C. Fantini, S. Wirtz, A. Nikolaev, R. Kiesslich, H. A. Lehr, P. R. Galle, and M. F. Neurath. 2005. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* 54(7):950-954.

Belz, E. E., J. S. Kennell, R. K. Czambel, R. T. Rubin, and M. E. Rhodes. 2003. Environmental enrichment lowers stress-responsive hormones in singly housed male and female rats. *Pharmacol Biochem Be* 76(3-4):481-486.

Benaroya-Milshtein, N., N. Hollander, A. Apter, T. Kukulansky, N. Raz, A. Wilf, I. Yaniv, and C. G. Pick. 2004. Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *Eur J Neurosci* 20(5):1341-1347.

Benefiel, A. C., W. K. Dong, and W. T. Greenough. 2005. Mandatory "enriched" housing of laboratory animals: The need for evidence-based evaluation. *ILAR J* 46(2):95-105.

Blanchard, D. C. and R. J. Blanchard. 2005. Stress and aggressive behaviors. In *Biology of Aggression*, R. J. Nelson, ed. Oxford: Oxford University Press.

Bradshaw, J. W. S. and S. L. Hall. 1999. Affiliative behaviour of related and unrelated pairs of cats in catteries: A preliminary report. *App Anim Behav Sci* 63(3):251-255.

Broadbear, J. H., G. Winger, J. E. Rivier, and J. H. Woods. 2004. Corticotropin-releasing hormone antagonists, astressin B and antalarmin: Differing profiles of activity in rhesus monkeys. *Neuropsychopharmacology* 29(6):1112-1121.

Canadian Council on Animal Care (CCAC). 1993. Guide to the Care and Use of Experimental Animals, 2nd Edition.

Chung, K. C., L. K. Kalliainen, S. V. Spilson, M. R. Walters, and H. M. Kim. 2002. The prevalence of negative studies with inadequate statistical power: An analysis of the plastic surgery literature. *Plast Reconstr Surg* 109(1):1-6.

Clark, J. M. 1997. Biocontainment facilities: Implications for animal care and welfare. In *Animal Alternatives, Welfare, and Ethics*, L. F. M. van Zutphen and M. Balls, eds. New York: Elsevier.

Clark, V. A. 2002. The prevalence of negative studies with inadequate statistical power: An analysis of the plastic surgery literature by Kevin C. Chung, MD, MS, Loree K. Kalliainen, MD, Sandra V. Spilson, MPH, Madonna R. Walters, MS, RN, Hyungjin Myra Kim, ScD. *Plast Reconstr Surg* 109(1):7-8.

Clough, G. 1982. Environmental effects on animals used in biomedical research. *Biol Rev* 57(Pt 3):487-523.

Coleman, K., L. A. Tully, and J. L. McMillan. 2005. Temperament correlates with training success in adult rhesus macaques. *Am J Primatol* 65(1):63-71.

Council of Europe. 2006. European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes-CETS no: 123. Appendix A (final version for adoption).

Cronin, G. M., P. R. Wiepkema, and J. M. van Ree. 1985. Endogenous opioids are involved in abnormal stereotyped behaviours of tethered sows. *Neuropeptides* 6(6):527-530.

Cudilo, E., H. Al Naemi, L. Marmorstein, and A. L. Baldwin. 2007. Knockout mice: Is it just genetics? Effect of enriched housing on fibulin-$4^{+/-}$ mice. *PLoS ONE* 2(2):e229.

Davenport, M. D., S. Tiefenbacher, C. K. Lutz, M. A. Novak, and J. S. Meyer. 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen Comp Endocr* 147(3):255-261.

Dean, S. W. 1999. Environmental enrichment of laboratory animals used in regulatory toxicology studies. *Lab Anim* 33(4):309-327.

DHHS (Department of Health and Human Services), National Institutes of Health, Office of Laboratory Animal Welfare. 2005. Guidance on Prompt Reporting to OLAW under the PHS Policy on Humane Care and Use of Laboratory Animals. NIH Guide for Grants and Contracts, Notice Number: NOT-OD-05-034. Available at: http://grants.nih.gov/grants/guide/notice-files/NOT-OD-05-034.html. Accessed January 30, 2008.

Dell, R. B., S. Holleran, and R. Ramakrishnan. 2002. Sample size determination. *ILAR J* 43(4):207-213.

Dirnagl, U. 2006. Bench to bedside: The quest for quality in experimental stroke research. *J Cereb Blood F Met* 26(12):1465-1478.

Duke, J. L., T. G. Zammit, and D. M. Lawson. 2001. The effects of routine cage-changing on cardiovascular and behavioral parameters in male Sprague-Dawley rats. *Contemp Top Lab Anim* 40(1):17-20.

Eaton, G. G., S. T. Kelley, M. K. Axthelm, S. A. Iliff-Sizemore, S. M. Shiigi. 1994. Psychological well-being in paired adult female rhesus *(Macaca mulatta). Amer J Primatol* 33:88-89.

FELASA Working Group Standardization of Enrichment. 2006. Working group Report. Available at www.lal.org.uk/pdffiles/FELASA_Enrichment_2006.pdf. Accessed January 30, 2008.

Festing, M. F. W. and D. G. Altman. 2002. Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR J* 43(4):244-258.

Festing, M. F. W., P. Diamanti, and J. A. Turton. 2001. Strain differences in haematological response to chloramphenicol succinate in mice: Implications for toxicological research. *Food Chem Toxicol* 39(4):375-383.

Festing, M. F. W., P. Overend, R. Gaines Das, C. Borja, and M. Berdoy. 2002. *The Design of Animal Experiments: Reducing the Use of Animals in Research through Better Experimental Design: Laboratory Animal Handbook Series #14*. London: RSM Press LTD. 1-112 pp.

Fontenot, M. B., E. E. Padgett III, A. M. Dupuy, C. R. Lynch, P. B. De Petrillo, and J. D. Higley. 2005. The effects of fluoxetine and buspirone on self-injurious and stereotypic behavior in adult male rhesus macaques. *Comparative Med* 55(1):67-74.

Fontenot, M. B., M. N. Wilkes, and C. S. Lynch. 2006. Effects of outdoor housing on self-injurious and stereotypic behavior in adult male rhesus macaques *(Macaca mulatta). J Am Assoc Lab Anim Sci* 45(5):35-43.

Garner, J. P. 2005. Stereotypies and other abnormal repetitive behaviors: Potential impact on validity, reliability, and replicability of scientific outcomes. *ILAR J* 46(2):106-117.

Garner, J. P. 2006. Perseveration and stereotypy: Systems-level insights from clinical psychology. In *Stereotypic Animal Behavior: Fundamentals and Applications to Welfare,* 2nd ed, G. Mason and J. Rushen, eds. Wallingford, UK: CAB International. 121-153 pp.

Gold, L. S., N. B. Manley, T. H. Slone, L. Rohrbach, and G. B. Garfinkel. 2005. Supplement to the Carcinogenic Potency Database (CPDB): Results of animal bioassays published in the general literature through 1997 and by the National Toxicology Program in 1997-1998. *Toxicol Sci* 85(2):747-808.

Gray, S. and J. L. Hurst. 1995. The effects of cage cleaning on aggression within groups of male laboratory mice. *Anim Behav* 49(3):821-826.

Green, T. A., B. J. Gehrke, and M. T. Bardo. 2002. Environmental enrichment decreases intravenous amphetamine self-administration in rats: Dose-response functions for fixed- and progressive-ratio schedules. *Psychopharmacology* 162(4):373-378.

Haemisch, A. and K. Gartner. 1994. The cage design affects intermale aggression in small groups of male laboratory mice: Strain specific consequences on social organization, and endocrine activations in two inbred strains (DBA/2J and CBA/J). *J Exp Anim Sci* 36(4-5):101-116.

Hawkins, P., L. M. Felton, P. L. Van Loo, M. Maconochie, D. J. Wells, N. Dennison, R. Hubrecht, and M. Jennings. 2006. Report of the 2005 RSPCA/UFAW Rodent Welfare Group Meeting. *Lab Anim* 35(9):10.

Hawkins, P., D. B. Morton, R. Bevan, K. Heath, J. Kirkwood, P. Pearce, L. Scott, G. Whelan, and A. Webb. 2004. Husbandry refinements for rats, mice, dogs and non-human primates used in telemetry procedures. Seventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement, Part B. *Lab Anim* 38(1):1-10.

Hendriksen, C. F. M. and D. B. Morton, eds. 1999. *Humane Endpoints in Animal Experiments for Biomedical Research.* London: Royal Society of Medicine.

Herman, J. P., M. M. Ostrander, N. K. Mueller, and H. Figueiredo. 2005. Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Prog Neuro-Psychoph* 29(8):1201-1213.

Hetts, S. 1991. Psychologic well-being: Conceptual issues, behavioral measures, and implications for dogs. *Vet Clin N Am-Small* 21(2):369-387.

Hobbs, B. A., W. Kozubal, and F. F. Nebiar. 1997. Evaluation of objects for environmental enrichment of mice. *Contemp Top Lab Anim* 36(3):69-71.

Hockly, E., P. M. Cordery, B. Woodman, A. Mahal, A. van Dellen, C. Blakemore, C. M. Lewis, A. J. Hannan, and G. P. Bates. 2002. Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. *Ann Neurol* 51(2):235-242.

Holy, T. E. and Z. Guo. 2005. Ultrasonic songs of male mice. *PLoS Biol* 3(12):e386.

Hubrecht, R. C. 1995. Housing, Husbandry and Welfare Provision for Animals Used in Toxicology Studies: Results of a UK Questionnaire on Current Practice. Potters Bar: Toxicology and Welfare Working Group.

Hubrecht, R. C. 1997. Comfortable quarters for laboratory dogs. In *Comfortable Quarters for Laboratory Animals,* V. Reinhardt, ed. Washington: Animal Welfare Institute. 63-74 pp.

Hugo, C., J. Seier, C. Mdhluli, W. Daniels, B. H. Harvey, D. Du Toit, S. Wolfe-Coote, D. Nel, and D. J. Stein. 2003. Fluoxetine decreases stereotypic behavior in primates. *Prog Neuro-Psychoph* 27:639-643.

Hurst, J. L., J. Fang, and C. J. Barnard. 1993. The role of substrate odours in maintaining social tolerance between male house mice *Mus musculus domesticus. Anim Behav* 45:997-1006.

Hurst, J. L., C. J. Barnard, C. M. Nevison, and C. D. West. 1997. Housing and welfare in laboratory rats: Welfare implications of isolation and social contact among caged males. *Anim Welfare* 6(4):329-347.

Hurst, J. L., 1998. Housing and welfare in laboratory rats: The welfare implications of social isolation and social contact among females. *Anim Welfare* 7(2):121-136.

ILAR Journal. 2006. Preparation of Animals for Use in the Laboratory. *ILAR J* 47(4):281-390.

Jaffer, F. A., P. Libby, and R. Weissleder. 2006. Molecular and cellular imaging of atherosclerosis: Emerging applications. *J Am Coll Cardiol* 47(7):1328-1338.

Jarrell, D. M., J. Camacho, D. Funk-Flavin, and S. M. Niemi. 2005. A new method for comprehensive, non-invasive physiological data recording in conscious macaques. *Contemp Top Lab Anim* 44(4):69.

Jennings, M., G. R. Batchelor, P. F. Brain, A. Dick, H. Elliott, R. J. Francis, R. C. Hubrecht, J. L. Hurst, D. B. Morton, A. G. Peters, R. Raymond, G. D. Sales, C. M. Sherwin, and C. West. 1998. Refining rodent husbandry: The mouse. *Lab Anim* 32(3):233-259.

Kaldi, I., M. Dittmar, P. Pierce, and R. E. Anderson. 2003. L-NAME protects against acute light damage in albino rats, but not against retinal degeneration in P23H and S334ter transgenic rats. *Exp Eye Res* 76(4):453-461.

Kessel, A. L. and L. Brent. 1998. Cage toys reduce abnormal behavior in individually housed pigtail macaques. *J Appl Anim Welf Sci* 1(3):227-234.

Kiess, W., A. Meidert, R. A. Dressendorfer, K. Schriever, U. Kessler, A. Konig, H. P. Schwarz, and C. J. Strasburger. 1995. Salivary cortisol levels throughout childhood and adolescence: Relation with age, pubertal stage, and weight. *Pediatr Res* 37(4 Pt 1):502-506.

Lawson, D. M., M. Churchill, and P. C. Churchill. 2000. The effects of housing enrichment on cardiovascular parameters in spontaneously hypertensive rats. *Contemp Top Lab Anim* 39(1):9-13.

Maier, S. F. and L. R. Watkins. 2005. Stressor controllability and learned helplessness: The roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neurosci Biobehav* 29(4-5):829-841.

Marai, I. F. M., A. A. M. Habeeb, and A. E. Gad. 2002. Rabbits' productive, reproductive, and physiological performance traits as affected by heat stress: A review. *Livest Prod Sci* 78(2):71-90.

Marashi, V., A. Barnekow, E. Ossendorf, and N. Sachser. 2003. Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Horm Behav* 43(2):281-292.

Mead, R. 1988. *The Design of Experiments: Statistical Principles for Practical Applications*. Cambridge: Cambridge University Press.

Meijer, M. K., R. Sommer, B. M. Spruijt, L. F. M. Van Zutphen, and V. Baumans. 2007. Influence of environmental enrichment and handling on the acute stress response in individually housed mice. *Lab Anim* 41:161-173.

Milligan, S. R., G. D. Sales, K. Khirnykh. 1993. Sound levels in rooms housing laboratory animals: An uncontrolled daily variable. *Physiol Behav* 53(6):1067-1076.

Moncek, F., R. Duncko, B. B. Johansson, and D. Jezova. 2004. Effect of environmental enrichment on stress related systems in rats. *J Neuroendocrinol* 16(5):423-431.

Morton, D. B. 2000. A systematic approach for establishing humane endpoints. *ILAR J* 41(2):40-42.

Morton, D. B. 1998. The importance of non-statistical design in refining animal experimentation. ANZCCART Facts Sheet. *ANZCCART News* 11 (2). Available at www.adelaide.edu.au/ANZCCART/publications/fs17.pdf. Accessed January 30, 2008.

Morton, D. B. and J. Hau. 2002. Welfare assessment and humane endpoints. In: *CRC Handbook of Laboratory Animal Science, Vol. 1: Essential Principles and Practices*, 2nd ed. J. Hau and G. L. Van Hoosier, Jr., eds. Boca Raton, FL: CRC Press. 457-486 pp.

Morton, D. B., M. Jennings, G. R. Batchelor, D. Bell, L. Birke, K. Davies, J. R. Eveleigh, D. Gunn, M. Heath, B. Howard, P. Koder, J. Phillips, T. Poole, A. W. Sainsbury, G. D. Sales, D. J. A. Smith, M. Stauffacher, and R. J. Turner. 1993. Refinements in rabbit husbandry: Second report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Lab Anim* 27(4):301-329.

Morton, D. B., P. Hawkins, R. Bevan, K. Heath, J. Kirkwood, P. Pearce, L. Scott, G. Whelan, and A. Webb. 2003. Refinements in telemetry procedures. Seventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement, Part A. *Lab Anim* 37(4):261-299.

National Health and Medical Research Council, Australian Government. 2004. Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition.

NRC (National Research Council). 1996. *Guide for the Care and Use of Laboratory Animals.* Washington: National Academy Press.

NRC. 2003a. *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research 2003.* Washington: The National Academies Press.

NRC. 2003b. *International Workshop on the Development of Science-based Guidelines for Laboratory Animal Care.* Washington: The National Academies Press.

NRC. 2006. *Guidelines for the Humane Transportation of Research Animals.* Washington: The National Academies Press.

Novak, M. A., J. H. Kinsey, M. J. Jorgensen, and T. J. Hazen. 1998. Effects of puzzle feeders on pathological behavior in individually housed rhesus monkeys. *Am J Primatol* 46(3):213-227.

Novak, M. A. 2003. Self-injurious behavior in rhesus monkeys: New insights into its etiology, physiology, and treatment. *Am J Primatol* 59(1):3-19.

Olsson, I. A. and C. M. Sherwin. 2006. Behaviour of laboratory mice in different housing conditions when allowed to self-administer an anxiolytic. *Lab Anim* 40(4):392-399.

Olsson, I. A. and K. Dahlborn. 2002. Improving housing conditions for laboratory mice: A review of 'environmental enrichment'. *Lab Anim* 36(3):243-270.

Passchier-Vermeer, W. and W. F. Passchier. 2000. Noise exposure and public health. *Environ Health Persp* 108(Suppl 1):123-131.

Patterson-Kane, E. G. 2002. Cage size preference in rats in the laboratory. *J Appl Anim Welf Sci* 5(1):63-72.

Perremans, S., J. M. Randall, L. Allegaert, M. A. Stiles, G. Rombouts, and R. Geers. 1998. Influence of vertical vibration on heart rate of pigs. *J Anim Sci* 76(2):416-420.

Phelps, E. A. and J. E. LeDoux. 2005. Contributions of the amygdala to emotion processing: From animal models to human behavior. *Neuron* 48(2):175-187.

Pinkert, C. A. 2003. Transgenic animal technology: Alternatives in genotyping and phenotyping. *Comparative Med* 53(2):126-139.

Poon, R. and I. Chu. 1999. Inflammatory cecal masses in patients presenting with appendicitis. *World J Surg* 23(7):713-716.

Prescott, M. J. and H. M. Buchanan-Smith. 2003. Training nonhuman primates using positive reinforcement techniques. *J Appl Anim Welf Sci* 6(3):157-161.

Reinhardt, V. 2003. Working with rather than against macaques during blood collection. *J Appl Anim Welf Sci* 6(3):189-197.

Riley, J. L., 3rd, M. E. Robinson, E. A. Wise, C. D. Myers, and R. B. Fillingim. 1998. Sex differences in the perception of noxious experimental stimuli: A meta-analysis. *Pain* 74(2-3):181-187.

Roberts, S. J. and M. L. Platt. 2005. Effects of isosexual pair-housing on biomedical implants and study participation in male macaques. *Contemp Top Lab Anim* 44(5):13-18.

Robinson, V., D. B. Morton, D. Anderson, J. F. A. Carver, R. J. Francis, R. Hubrecht, E. Jenkins, K. E. Mathers, R. Raymond, I. Rosewell, J. Wallace, and D. J. Wells. 2003. Refinement and reduction in production of genetically modified mice. *Lab Anim* 37(Suppl 1).

Roy, V., C. Belzung, C. Delarue, and P. Chapillon. 2001. Environmental enrichment in BALB/c mice: Effects in classical tests of anxiety and exposure to a predatory odor. *Physiol Behav* 74(3):313-320.

Russell, W. M. S. and R. L. Burch. 1959. *The Principles of Humane Experimental Technique*. London: Methuen.

Sales, G. D., R. Hubrecht, A. Peyvandi, S. Milligan, and B. Shield. 1997. Noise in dog kenneling: Is barking a welfare problem for dogs? *Appl Anim Behav Sci* 52(3-4):321-329.

Sales, G. D., S. R. Milligan, and K. Khirnykh. 1999. Sources of sound in laboratory animal environment: A survey of the sounds produced by procedures and equipment. *Anim Welfare* 8(2):97-115.

Sapolsky, R. M. 1994. *Why Zebras Don't Get Ulcers: A Guide to Stress, Stress Related Diseases, and Coping*. New York: WH Freeman.

Schapiro, S. J., J. E. Perlman, and B. A. Boudreau. 2001. Manipulating the affiliative interactions of group-housed rhesus macaques using positive reinforcement training techniques. *Am J Primatol* 55(3):137-149.

Schrijver, N. C., N. I. Bahr, I. C. Weiss, and H. Würbel. 2002. Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. *Pharmacol Biochem Be* 73(1):209-224.

Sedcole, J. R. 2006. Experimental design: Minimizing the number of subjects that suffer may not mean minimizing total suffering. *Anim Behav* 71(3):735-738.

Sharp, J., T. Azar, and D. Lawson. 2005. Effects of a cage enrichment program on heart rate, blood pressure, and activity of male Sprague-Dawley and spontaneously hypertensive rats monitored by radiotelemetry. *Contemp Top Lab Anim* 44 (2):32-40.

Sharp, J. and D. Lawson. 2003. Does cage size affect heart rate and blood pressure of male rats at rest or after procedures that induce stress-like responses? *Contemp Top Lab Anim* 42(3):8-12.

Sharp, J. L., T. Zammit, T. Azar, and D. M. Lawson. 2002b. Stress-like responses to common procedures in male rats housed alone or with other rats. *Contemp Top Lab Anim* 41(4):8-14.

Shaw, R., M. F. W. Festing, I. Peers, and L. Furlong. 2002. Use of factorial designs to optimize animal experiments and reduce animal use. *ILAR J* 43(4):223-232.

Sherwin, C. M. 2004. The influences of standard laboratory cages on rodents and the validity of research data. *Anim Welfare* 13(Suppl):9-15.

Sherwin, C. M. and I. A. S. Olsson. 2004. Housing conditions affect self-administration of anxiolytic by laboratory mice. *Anim Welfare* 13(1):33-38.

Shoji, R., U. Murakami, and T. Shimizu. 1975. Influence of low-intensity ultrasonic irradiation on prenatal development of two inbred mouse strains. *Teratology* 12(3):227-231.

Soothill, J. S., D. B. Morton, and A. Ahmad. 1992. The HID_{50} (hypothermia-inducing dose 50): An alternative to the LD_{50} for measurement of bacterial virulence. *Int J Exp Pathol* 73:95-98.

Sterne, J. A. C. and G. D. Smith. 2001. Sifting the evidence—What's wrong with significance tests? *Brit Med J* 322(7280):226-231.

Stokes, W. S. 2000. Reducing unrelieved pain and distress in laboratory animals using humane endpoints. *ILAR J* 41(2):59-61.

Taylor, D. K., T. Bass, G. S. Flory, and F. C. Hankenson. 2005. Use of low-dose chlorpromazine in conjunction with environmental enrichment to eliminate self-injurious behavior in a rhesus macaque *(Macaca mulatta)*. *Comparative Med* 55(3):282-288.

Tiefenbacher, S., M. D. Davenport, M. A. Novak, A. L. Pouliot, and J. S. Meyer. 2003. Fenfluramine challenge, self-injurious behavior, and aggression in rhesus monkeys. *Physiol Behav* 80(2-3):327-331.

Tiefenbacher, S., M. A. Fahey, J. K. Rowlett, J. S. Meyer, A. L. Pouliot, B. M. Jones, and M. A. Novak. 2005. The efficacy of diazepam treatment for the management of acute wounding episodes in captive rhesus macaques. *Comparative Med* 55(4):387-392.

Tomei, F., M. G. Ruffino, E. Tomao, T. P. Baccolo, M. V. Rosati, and F. Strollo. 2000. Acute experimental exposure to noise and hormonal modifications. *J Environ Sci Heal A* 35:537-555.

Tsai, P. P., U. Pachowsky, H. D. Stelzer, and H. Hackbarth. 2002. Impact of environmental enrichment in mice: Effect of housing conditions on body weight, organ weights and haematology in different strains. *Lab Anim* 36(4):411-419.

Turner, P. V., K. L. Smiler, M. Hargaden, and M. A. Koch. 2003. Refinements in the care and use of animals in toxicology studies—regulation, validation, and progress. *Contemp Top Lab Anim* 42(6):8-15.

Vahl, T. P., Y. M. Ulrich-Lai, M. M. Ostrander, C. M. Dolgas, E. E. Elfers, R. J. Seeley, D. A. D'Alessio, and J. P. Herman. 2005. Comparative analysis of ACTH and corticosterone sampling methods in rats. *Am J Physiol Endoc M* 289(5):E823-828.

USDA (U.S. Department of Agriculture). 2005. 9CFR2.31. Code of Federal Regulations, title 9, Volume 1, part 2, subpart C, section 2.31 (d) (1) (i): Institutional Animal Care and Use Committee. Available at: http://www.aphis.usda.gov/animal_welfare/downloads/awr/9cfr2.31.txt. Accessed January 30, 2008.

Van Loo, P. L., E. Van der Meer, C. L. J. J. Kruitwagen, J. M. Koolhaas, L. F. Van Zutphen, and V. Baumans. 2004. Long-term effects of husbandry procedures on stress-related parameters in male mice of two strains. *Lab Anim* 38(2):169-177.

Van Loo, P. L., H. A. Van de Weerd, L. F. Van Zutphen, and V. Baumans. 2004. Preference for social contact versus environmental enrichment in male laboratory mice. *Lab Anim* 38(2):178-188.

Wasowicz, M., C. Morice, P. Ferrari, J. Callebert, and C. Versaux-Botteri. 2002. Long-term effects of light damage on the retina of albino and pigmented rats. *Invest Ophth Vis Sci* 43(3):813-820.

Waterton, J. C., B. J. Middleton, R. Pickford, C. P. Allott, D. Checkley, R. A. Keith. 2000. Reduced animal use in efficacy testing in disease models by the use of sequential experimental designs. In *Progress in the Reduction, Refinement and Replacement of Animal Experimentation*, M. Balls, A.-M. van Zeller, and M. Halder, eds. Oxford: Elsevier Science.

Weed, J. L. and J. M. Raber. 2005. Balancing animal research with animal well-being: Establishment of goals and harmonization of approaches. *ILAR J* 46(2):118-128.

Weissleder, R. 2006. Molecular imaging in cancer. *Science* 312(5777):1168-1171.

Weld, K. P., J. A. Mench, R. A. Woodward, M. S. Bolesta, S. J. Suomi, and J. D. Higley. 1998. Effect of tryptophan treatment on self-biting and central nervous system serotonin metabolism in rhesus monkeys *(Macaca mulatta). Neuropsychopharmacology* 19(4):314-321.

Würbel, H. 2000. Behaviour and the standardization fallacy. *Nat Genet* 26(3):263.

5

Topics for Further Investigation and Recommendations

RESEARCH DIRECTIONS

The constant generation of technological and scientific advances provides us with the means to answer scientific inquiries in all fields with greater accuracy and precision. One can therefore reasonably expect further reductions in the causes of laboratory animal distress without compromising scientific or regulatory principles, if the scientific community continues to approach this subject with the diligence, imagination, and compromise demonstrated to date. Interdisciplinary projects and translational research foci in particular offer many avenues to explore toward identifying and reducing distress in research animals. The following suggestion list is the distillate of many diverse opinions rather than firm directions for the future and is presented in no particular order for the reader's consideration:

- Are there molecular or other markers of distress (e.g., fMRI and PET scans of blood flow through the brain) that reflect an animal's physiological and perhaps even mental state (Gingrich 2006)? Is it possible to obtain those markers easily and harmlessly? Can they indicate the relative predisposition to distress of different species, different genetic strains of the same species, or even individual animals? If the answer is yes to any of these questions, such markers would provide powerful new tools for the intertwined fields of distress research and animal welfare research.
- Tools (such as microarrays) used in genomics and proteomics research could contribute toward an integrated picture of the

physiology and pathophysiology of stress and distress. Modeling this knowledge across species and strains would both enlarge our understanding of distress and enable translational approaches to human diseases as well as improvements in animal welfare. The absence of a consensus definition of distress affects the evaluation of distress and its impact on animal welfare in veterinary, scientific, and legislative contexts; integrative research approaches could be immensely helpful in this area.

- The development of possible distress predictors could serve as the basis for a predictive scoring system for laboratory animals, similar to the system used for the severity of illness in human intensive care units (Knaus et al. 1985, 1991). The ability to perform standardized, quantitative, and comprehensive evaluations of animals in poor health or in distress would enable teams to make decisions about continued treatment versus euthanasia faster and with greater consensus. Such a system would further assist important decisions about the adoption and/or refinement of humane endpoints before the initiation of experiments, especially if the clinical assessment is validated through postmortem examinations. As shown in the Appendix, score sheets can be used to identify any number of abnormal signs, some of which will help diagnose the cause of the abnormality or will be relevant to individual research protocols. While some of the clinical observations and test results would be common among various experiments, the creation of a standardized predictive scoring system for distress is predicated upon a definition of distress and identification of the crucial parameters that accompany its clinical presentation.
- New research could delineate the mechanisms of possible associations between stress/distress and disease behaviors or abnormal behaviors (e.g., stereotypies). Collaborative investigation is necessary to identify the neural processes, systems, and pathways that regulate active or passive coping in stressful situations, "permit" development of distress, or enable abnormal behaviors. Because stereotypies may adversely affect research outcomes and lead to invalidated studies and the need for repetitions, research is essential to determine, among other things, whether their presence could serve as a reliable indicator of animal welfare.
- With the genetic manipulation of increasing numbers of animal species and the creation of new animal types (e.g., "humanized" mice) to better mimic human pathophysiology and disease, it is crucial to have a deeper and complete understanding of how the characteristics of an organism (such as gender or age) or its manipulated genotype can influence the development of distress (for an example

of behavioral assessment of transgenic mice see Appendix). Moreover, the improvements in husbandry that support the successful creation of transgenic and genetically modified (GM) colonies, could provide clues for refinement of breeding and husbandry procedures in the non-GM laboratory animal world. This knowledge will enable further investigation into the conditions under which stress or distress do (or do not) alter the course of a disease.

- Should IACUCs, preclinical study safety officers, and scientific journal editors establish criteria by which historically acceptable control animals would suffice for statistical comparisons in certain situations? If otherwise scientifically and methodologically valid (more information on the challenges of using historical controls in Chapter 4), such a change would reduce the number of control animals used in potentially or intentionally distress-inducing protocols. Standardization and awareness of key *Three Rs*-related words and concepts among editors and reviewers would promote their application, especially in refinements. In a similar spirit, could the often useful but underappreciated approach of humans serving as the "animal" model be similarly informative for animal distress situations (Niemi 2006)? For example, could progress in human psychopharmacology enable the extrapolation of new drugs or indications to prevent or relieve distress in laboratory animals?
- It is essential to continue the review of currently approved euthanasia methods, discussion of the duration of an animal's distress before loss of consciousness, and research on the applicability of the *Three Rs.* For example, what refinements in the euthanasia of large populations of animals (e.g., mice) would be nondistressing to the animal as well as cost-effective and safe? The use of high concentrations of carbon dioxide is similarly contentious, as it is perceived by some as likely to be painful while it is also clearly aversive. As a euthanasia agent it may also be distressful even though consciousness probably ceases in less than a minute. Furthermore, debate has focused on the use of cervical dislocation, decapitation, and neck cutting as more appropriate methods of euthanasia with respect to the time needed for the animal to lose consciousness (Hawkins et al. 2006; EFSA 2006; AVMA 2007). Last, scientific interventions should also address the serious emotional effects on personnel who habitually perform euthanasia.
- Are there established parameters for a truly optimal husbandry system for each species of laboratory animal and for the genetic lines within those species? Animal care facility managers may wonder, for example, if it is more humane to disturb mice that normally sleep in the daytime for daily health assessments versus observ-

ing them passively, even though the latter approach might result in missing something serious. While there exists a growing body of scientific evidence (for example see Bayne et al. 2002; EEC 1986; Kaliste 2004; Morton 2002), it is important to approach continuing attempts to establish what is in the best interests of animals with rigorous scientific interdisciplinary methods. Even experts such as veterinarians, ethologists, and animal welfare scientists have to guard against the twin traps of anthropomorphism and anthropocentrism when interpreting such data (Bradshaw and Casey 2007).

- The use of experimental designs currently used for human research may offer new insights and opportunities in studies that depend on laboratory animals and should be further explored. Epidemiological approaches can help identify management and biological factors involved in the etiology of problem behaviors (McGreevy et al. 1995; Nicol et al. 2003), and matched-pair designs may allow for smaller sample sizes because of their powerful capacity (Würbel and Garner 2007).

RECOMMENDATIONS

The following recommendations are the intellectual product of this Committee's deliberations; however, we acknowledge some overlap with the recent report of the Working Group on Animal Distress in the Laboratory (Brown et al. 2006).

1. **The *Three Rs* (refinement, reduction, and replacement) should be the standard for identifying, modifying, avoiding, and minimizing most causes of distress in laboratory animals.** While research on distress and methods of alleviating distress (e.g., the development of anesthesia or analgesia) may unavoidably cause animal suffering, the optimum goal of research and veterinary teams should be to reduce and alleviate distress in laboratory animals to the minimum necessary to achieve the scientific objective.

2. **Protocols should include efforts to improve housing and husbandry conditions through the judicious employment of strategies for enrichment, animal training, and socialization.** Well-trained, competent, and attentive research and animal care personnel are crucial in providing relief from unintended distress that originates from the care and use of laboratory animals.

3. **Institutional support for and embrace of a commitment to animal welfare of the laboratory animals is essential. Veterinarians and animal care personnel who work with research animals on a daily basis should have adequate time and contact with the animals to properly evaluate their well-being.** Funding for training programs is crucial to the training and development of specialized laboratory animal veterinarians and animal behaviorists and should increase, because in addition to such objective measurements as weight loss or lack of grooming, clinical judgment is vital to effective assessments of stress and distress.

4. **Appropriate statistical methodologies are an essential tool for the avoidance, minimization, and alleviation of distress.**

5. **There should be a clearinghouse (or some other venue such as a website or a specialized peer-reviewed journal) for publication of research on the effects of enrichment strategies on parameters such as physiology, distress, and endpoints for all laboratory animals** (one useful example is the Primate Enrichment Database hosted by the Animal Welfare Institute).[1] Although a variety of journals (such as *Lab Animal, Applied Animal Behaviour Science, Animal Welfare, Laboratory Animals, Contemporary Topics in Laboratory Animal Science, Comparative Medicine*) publish research pertaining to animal welfare, the highly specialized nature of the field makes it difficult for the larger scientific community to remain informed about recent advances and ongoing debates. Biomedical research journals should be more open to submissions from scientists whose research focuses on animal welfare issues so that concerns about research interference or unjustified expenses can be debated on scientific, ethical, or regulatory grounds.

6. Obtaining funding for welfare research is often difficult, especially when project applications compete against other fields of science due to lack of an appropriate/separate research oversight body. In the United Kingdom the funds available for welfare research have increased dramatically with the founding of the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).[2] In the United States, the National Institutes of Health, Environmental Protection Agency, and other federal institutions have occasionally provided funding to develop or validate

[1] http://www.awionline.org/SearchResultsSite/enrich.aspx.
[2] NC3Rs website: www.nc3rs.org.uk.

nonanimal or nonvertebrate alternatives. Funding for laboratory animal welfare research, however, is usually available only in small amounts from nongovernmental organizations such as the Animal Welfare Institute, the Johns Hopkins Center for Alternatives to Animal Testing, the American College of Laboratory Animal Medicine, and the American Association for Laboratory Animal Science. **Given the impact of better animal welfare on science as well as the growing public interest in the treatment of laboratory animals, federal agencies and large foundations that support biomedical and behavioral research should make funds available specifically for the avenues of investigation listed above and for other related topics.**

7. **Animal welfare scientists and researchers and scientists who use animal models should communicate with each other more frequently in order to compare objectives and progress and to identify opportunities for collaboration.** Neutral groups and/or other established research and science policy entities can provide platforms and venues for such exchanges.

REFERENCES

AVMA (American Veterinary Medical Association). 2007. 2007 AVMA Guidelines on Euthanasia.

Bayne, K. A. L., J. A. Mench, B. V. Beaver, and D. B. Morton. 2002. Laboratory Animal Behavior. In: *Laboratory Animal Science,* ACLAM series 2nd Ed. New York: Academic Press. 1240-1264 pp.

Bradshaw, W. S. and R. A. Casey. 2007. Anthropomorphism and anthropocentrism as influences in the quality of life of companion animals. *Anim Welf* 16 (suppl): in press.

Brown, M., L. Carbone, K. M. Conlee, M. S. Dawkins, I. J. Duncan, D. Fraser, G. Griffin, V. A. Hampshire, L. A. Lambert, J. A. Mench, D. Morton, J. Richmond, B. E. Rollin, A. N. Rowan, M. L. Stephens, and H. Würbel. 2006. Report of the Working Group on Animal Distress in the Laboratory. *Lab Animal* 35(8):26-30.

EEC (European Economic Commission). 1986. Council Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes. Available at: ec.europa.eu/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf. Accessed August 2007.

EFSA (European Food Safety Authority Scientific Panel on Animal Health and Welfare). 2006. The welfare aspects of the main systems of stunning and killing applied to commercially farmed deer, goats, rabbits, ostriches, ducks, geese and quail. *EFSA J* 326:1-18.

Gingrich, J. A. 2006. Oxidative stress is the new stress. *Nat Med* 11(12):1281-1282.

Hawkins, P., L. M. Felton, P. L. Van Loo, M. Maconochie, D. J. Wells, N. Dennison, R. Hubrecht, and M. Jennings. 2006. Report of the 2005 RSPCA/UFAW Rodent Welfare Group Meeting. *Lab Anim* 35(9):10.

Hawkins, P., L. Playle, H. Golledge, M. Leach, R. Banzett, A. Coene, J. Cooper, P. Danneman, P. Flecknell, R. Kirkden, L. Niel, and M. Raj. 2006. Newcastle Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals. Available at: www.nc3rs.org.uk/downloaddoc.asp?id=416&page=292&skin=0. Accessed January 30, 2008.

Kaliste, E., ed. 2004. *The Welfare of Laboratory Animals*. Dordrecht/London: Kluwer Academic Publishers.

Knaus, W. A., J. E. Zimmerman, D. P. Wagner, E. A. Draper, and D. E. Lawrence. 1985. APACHE II—A severity of disease classification system. *Crit Care Med* 13:818-829.

Knaus, W. A., D. P. Wagner, E. A. Draper, J. E. Zimmerman, M. Bergner, P. G. Bastos, C. A. Sirio, D. J. Murphy, T. Lotring, A. Damiano, et al. 1991. The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults. *Chest* 100(6):1619-1636.

McGreevy, P. D., P. J. Cripps, N. P. French, L. E. Green, and C. J. Nicol. 1995. Management factors associated with stereotypic and redirected behavior in the thoroughbred horse. *Equine Vet J* 27(2):86-91.

Morton, D. B. 2002. Behaviour of rabbits and rodents. In *The Ethology of Domestic Animals—An Introductory Text,* P. Jensen, ed. Oxford: CABI Wallingford. 193-209 pp.

Nicol, C. J., C. Potzsch, K. Lewis, and L. E. Green. 2003. Matched concurrent case-control study of risk factors for feather pecking in hens on free-range commercial farms in the UK. *Br Poultry Sci* 44(4):515-523.

Niemi, S. M. 2006. Making comparative medicine more so. *J Am Vet Med Assoc* 228(2):199-200.

Weisstaub, N. V., M. Zhou, A. Lira, E. Lambe, J. Gonzalez-Maeso, J. P. Hornung, E. Sibille, M. Underwood, S. Itohara, W. T. Dauer, M. S. Ansorge, E. Morelli, J. J. Mann, M. Toth, G. Aghajanian, S. C. Sealfon, R. Hen, and J. A. Gingrich. 2006. Cortical 5-HT$_{2A}$ receptor signaling modulates anxiety-like behaviors in mice. *Science* 313(5786):536-540.

Würbel, H. and J. P. Garner. 2007. Refinement of rodent research through environmental enrichment and systematic randomization. *NC3Rs* 9:1-9.

Appendix

Tools to Monitor and Assess Health Status and Well-Being in Stress and Distress

The following pages contain ethograms, various types of scoring sheets, clinical assessments, and behavioral observations applicable to laboratory animals. As stated in the report, the interpretation of physiologic measurements as indicators of stress, distress, or welfare status is relative and does not always point to direct or straightforward links. Because little is known about behavioral changes directly attributed to stress and even less about distress, recognizing stress and distress in laboratory animals based on behavioral changes remains a significant challenge to investigators and animal care staff. Recognition of distress should be derived from intimate knowledge of the species' or strain's normal behavior and may be based on (1) clinical signs and/or (2) significant deviation from the expected behavioral repertoire. As a rule, when the expected repertoire of physiologic behaviors is absent or modified, an investigation into the reasons for the change is necessary.

Some clinical signs (e.g., changes in temperature, respiration, feeding behavior) indicate an abrupt onset of distress while others (e.g., weight loss) develop over a longer period of time and may serve as warnings. A thorough clinical examination with references to baseline effects of age, gender, genotype, etc., is necessary to establish the presence of distress, while an abrupt and marked change in behavior lasting more than a few days may also indicate a disease state. Although normal behaviors may sometimes be characterized simply by a lack of atypical behavior, such as stereotypic (i.e., repetitive) or self-injurious behavior, some species and strain differences are not always easy to discern, and further complications

are introduced by gender, age, physiological state, genetics, and genetic modification of the animals.

The first three tables below contain behavioral categories and descriptions of physiologic activities in which rhesus macaques, common marmosets, and rabbits engage. In order to determine what kind of behavior it is that an animal exhibits, one needs to be knowledgeable in the ethology and husbandry of the species in question. For example, aggression may be a signal for fear or pain, but may also be observed in lactating mothers protecting their nest. Determining the variation of the behavior from normalcy is a matter of training, studying, and observation.

TABLE A-1 An ethogram for *Macaca mulatta* (rhesus macaque)

Behavioral Categories	Recorded Behavior	Definitions
Aggressive behaviors	Facial threat displays	Open mouth face ± bared teeth or vocalization
	Aggressive approach	Stiff approach, attacking run
	Physical aggression	Slap, grab, biting, or wrestling
Submissive behaviors	Facial submissive display	Bared teeth grin ± vocalization
	Avoidance	Avoid, flee, leave, displaced
	Active appeasement	Groom present, lip smacking
Affiliative behaviors	Affiliative contact	Contact sit (within arms reach), embrace, touch
	Passive grooming	Being groomed
	Active grooming	Grooming other animal
Sexual behaviors	Sexual contact	Genital present/inspection, mounting
Appetitive behaviors	Foraging	Food search, eating, drinking
Other activities	Active	Locomotion, enrichment use, self-grooming
Abnormal behaviors	Abnormal behaviors	Stereotypies, autoaggression
Inactive	Inactive	Lying, huddling, sitting, sleeping
Vigilance	Monitoring others	Visually following other individuals

Reprinted from Augustsson, A. and J. Hau. 1999. A simple ethological monitoring system to assess social stress in group-housed laboratory rhesus macaques. *J Med Primatol* 28:84-90.

TABLE A-2 An ethogram for *Callithrix jacchus* (common marmoset)

Behavior	Code	Definition
Agonism		
Tufts-flick	TF	Rapid back-and-forth movement of ear tufts
Frown	FR	Lower eyebrows, furl brow, and turn down corners of mouth while staring
Cuff	CU	Swift, superficial blow or scratch performed aggressively
Chase	CH	Pursue partner, with one or both animals exhibiting aggression and/or submission (not play)
Fight	FI	Grapple aggressively with partner(s), involving biting, clawing, and wrestling
Attack	AT	Lunge at or pounce on partner aggressively; may or may not result in fight
Snap bite	SB	Direct a single short, sharp bite at partner
Submit	SU	Flatten ear tufts and/or facial grimace (partially open mouth with corners of mouth retracted, exposing lower and sometimes upper teeth) and/or slit eyes (eyelids half closed)
Continuous submit	CS	Continuous submit; start scoring after 5 sec
Retreat	RE	Starting from a stationary position, move at least one body length away from another animal within 1 sec of the other animal establishing proximity (within 10 cm)
Play		
Play	PL	Two or more animals lunge, grapple, wrestle, or chase for at least 1 sec in absence of aggression or intense submission; play face may or may not be present
Solicit play	SP	Direct play face toward, pounce on, or initiate grapple with partner, in absence of ongoing play with partner
Play face	PF	Open mouth without retraction of the lips
Join play	JP	Join ongoing play bout between two or more partners
End play	EP	Discontinue all social play for ≥ 3 sec
Social play	SO	Social interactions involving non-aggressive physical contact with other individuals; high activity
Infant-associated behaviors		
Climb on	ON	Climb onto any part of partner's body so that all four limbs are on partner
Solicit climb on	SC	Position body directly above infant and/or pull infant onto body; may or may not result in infant climbing onto partner's body
Climb off	OF	Voluntary climb off partner's body after having all four limbs on partner
Push off/reject	PO	Prevent juvenile from climbing onto body, or rub or otherwise force juvenile off body
Nurse	NU	Have mouth on female's nipple for ≥ 1 sec
End nursing	EN	Discontinue nursing posture

continued

TABLE A-2 Continued

Behavior	Code	Definition
Other social behaviors		
Sniff/nuzzle	SN	Orient face against or toward partner, excluding anogenital region
Anogenital inspect	AI	Orient face against or toward anogenital region of partner, or use hands or mouth to investigate anogenital region of partner; includes anogenital groom
Groom	GR	Use hands and/or mouth to pick through fur and/or mouth of partner, excluding anogenital region
Sexual solicit	SS	Stare at partner with ear tufts flattened and eyes slit
Mount	MO	Climb on partner's back from behind and grip partner around waist and legs; may be accompanied by pelvic thrusting
Initiate huddle	IH	Establish passive, torso-torso body contact with partner, with both animals remaining stationary and in passive contact for at least 3 sec
Leave huddle	LH	Terminate huddle after at least 3 sec of passive, torso-torso body contact during which both partners remained stationary
Object steal	OS	Take any nonfood object from hands or mouth of partner
Attempt object steal	AO	Attempt but fail to take nonfood object from hands or mouth of partner
Food-associated behaviors		
Food steal	ST	Take any food from hands or mouth of partner
Attempt food steal	AF	Attempt but fail to take food from hands or mouth of partner
Share food	SH	Eat from a food source from which partner is simultaneously eating or that partner is occupying without removing any food from partner's mouth or hands
New food	NF	Eat from a food source that no other animal is currently holding, eating from, or occupying
Individual behaviors		
Bristle strut	BS	Arching posture and/or strut locomotion and/or general piloerection
Scent mark	SM	Rub or drag anogenital, suprapubic, or sternal region along substrate, object, or partner
Genital present	GP	Raise tail to expose genitals
Object manipulation	OM	Sniff, bite, chew, gouge, handle, pounce on, grapple with, or otherwise manipulate inanimate object, excluding food items and water bottle, for at least 1 sec

Written by Lissa Pabst. From *Primate Info Net*, Library and Information Service, National Primate Research Center, University of Wisconsin-Madison. Available at: http://pin.primate.wisc.edu/callicam/ethogram.html.

TABLE A-3 An ethogram for rabbits

Common rabbit postures, behaviors, and vocalizations

Purring or teeth purring—A sound made by lightly and quickly grinding/vibrating the teeth as the whiskers quiver; a sign of contentment

Oinking or honking—A sound made to gain food or attention or during courtship

Clicking—A happy sound often made after a welcomed treat is given

Wheezing or sniffing—Nasal sounds made by 'talkative' rabbits; can be distinguished from abnormal respiratory sounds because they are intermittent and stimulated by interaction with the rabbit

Whimpering or low squealing—A fretting noise that is made when one picks up a rabbit that is reluctant to be handled; made often by pregnant and pseudopregnant does

Chinning—Rubbing the secretions from the scent glands under the chin on inanimate objects and people to mark possession. Glands are more developed in males than females

Nudging or nuzzling—The nose is used to nudge a person's hand or foot, or the rabbit may pull on a pant leg to signal a desire for attention. When enough petting has been done the rabbit may push the hand away

Head shaking, ear shaking, body shudder—A shake of the head or body in response to an annoying smell or unwanted handling; often occurs as the rabbit settles down and becomes relaxed enough to begin eating and grooming

Courting or circling—A sexual or social behavior whereby a rabbit circles another rabbit or the feet of a human while softly honking

Scratching at the floor—A rabbit may scratch at the floor with its forepaws in order to get a person's attention or to be picked up

Nipping—Not always done in anger, this can mean 'move over' or 'put me down'

Presentation—The head is extended forward with the feet tucked under the body and the chin placed on the floor in order for the rabbit to present itself as subordinate for petting from humans or to be groomed by another rabbit

Flattening—A fear response wherein the rabbit flattens its abdomen onto the floor with ears laid back against the head; the eyes may be bulging

Thumping—A sharp drumming of the hind feet as a warning or an alert to other rabbits of danger; often accompanied by dilation of the pupils and seeking refuge

Teeth grinding—A slower, louder teeth crunching, sometimes seen with bulging of the eyes and usually indicating discomfort, pain, or illness

Snorting or growling—A warning sound, either hissing or a short barking growl, that occurs with aggression or fear and is often seen with the ears flattened against the head and the tail up and in the grunt-lunge-bite sequence

Isolation—When a rabbit that normally seeks attention from its mates and human companions isolates itself and is less active. Such a rabbit should be checked for illness

Kicking—If a rabbit feels insecure when being picked up it will kick violently in an effort to escape. The hindquarters *must* be supported to prevent trauma to the spine or legs. A rabbit should be placed hind-end first into a cage in order to help prevent injuries caused by kicking

Aggression—Strained, upright stance with tail stretched out and ears laid back in defensive posture; the rabbit may also kick high and backwards

Loud, piercing scream—Similar to a human baby crying; signaling pain and fear, as when the rabbit is caught by a predator

Scanning—A rabbit with impaired vision may move its head from side to side to scan the area around it

Reprinted with permission from Macmillan Publishers Ltd: [Lab Animal] (Mayer 2007), copyright (2007). Mayer J. Use of behavioral analysis to recognize pain in small mammals. *Lab Anim* 36(6):43-48.

Other useful references for common normal behaviors of various laboratory animals include:

- Whishaw, I. Q. and B. Kolb, eds. 2005. *The Behavior of the Laboratory Rat: A Handbook with Tests*. New York: Oxford University Press.
- Pellis, S. M. and V. C. Pellis. 1987. Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat *Rattus norvegicus*. *Aggressive Behav* 13:227-242.
- Bassett, L., H. M. Buchanan-Smith, and J. McKinley. 2003. Effects of training on stress-related behavior of the common marmoset (*Callithrix jacchus*) in relation to coping with routine husbandry procedures. *J Appl Anim Welf Sci* 6(3):221-233.
- Stevenson, M. F. and T. B. Poole. 1976. An ethogram of the common marmoset (*Callithrix jacchus jacchus*): General behavioural repertoire. *Anim Behav* 24:428-451.
- Stone-Sade, D. 1973. An ethogram for rhesus monkeys. I. Antithetical contrasts in posture and movement. *Am J Phys Anthropol* 38(2):537-542.
- Universities Federation for Animal Welfare. 1995. An ethogram for behavioural studies of the domestic cat (*Felis silvestris catus* L.) of the UK. Cat Behaviour Working Group. Hertfordshire, England: Universities Federation for Animal Welfare.
- Morton, D. B. 2002. Behaviour of rabbits and rodents. In *The Ethology of Domestic Animals—An Introductory Text*, P. Jensen, ed. Oxford: CABI Wallingford Oxford. 193-209 pp.
- Bayne, K. A. L., J. A. Mench, B. V. Beaver, and D. B. Morton. 2002. Laboratory Animal Behavior. In *Laboratory Animal Science*, ACLAM series, 2nd ed. New York: Academic Press. 1240-1264 pp.
- Latham, N. and G. Mason. 2004. From house mouse to mouse house: The behavioural biology of free-living *Mus musculus* and its implications in the laboratory. *Appl Anim Behav Sci* 86:261-289.
- Bothe, G. M. W., V. J. Bolivar, M. J. Vedder, and J. G. Geistfeld. 2004. Genetic and behavioral differences among five inbred mouse strains commonly used in the production of transgenic and knockout mice. *Genes Brain Behav* 3:149-157.
- Fox, M. 1971. *Behaviour of Wolves, Dogs, and Related Canids*. New York: Harper & Row.
- Leyhausen, P. 1979. *Cat Behavior: The Predatory and Social Behavior of Domestic and Wild Cats*. Barbara A. Tonkin translation. New York: Garland STMP Press.
- Foster, H. L., J. D. Small, and J. G. Fox, eds. 1981. *The Mouse in Biomedical Research, vol. 1-4*. New York: Academic Press.

• Dixon, A. K. and H. U. Fisch. 1998. Animal models and ethological strategies for early drug testing in humans. *Neurosci Biobehav Rev* 23(2):345-358.

As stated in various places in the report, an effective assessment of distress is predicated upon solid knowledge of physiologic behaviors for each species and careful observation. In this respect, clinical and behavioral analysis of distress follows the investigative guidelines to determine the cause of any clinical symptomatology or pathology. Similarly, the goal of this exercise would be to remove, alleviate, or minimize the cause of distress (if doing so does not conflict with the research protocol) and support the animal in order to help it recover (see decision-making algorithm at the end of Chapter 4). The approach should integrate information from multiple behavioral and physiological parameters and should involve a team approach that includes researchers, veterinarians, and animal caretakers/technicians, as distress levels will vary in relation to the species, husbandry conditions, and experimental protocol as well as with each individual animal. The Committee points out that differential diagnosis of signs (clinical and behavioral) attributed to pain, sickness, or distress is quite difficult and requires careful observation and clinical skills. The following tables showcase the overlapping clinical signs and abnormal behaviors associated both with distress and/or pain in various animal species.

TABLE A-4 Species-specific clinical signs indicating pain, distress, or discomfort in experimental animals

Species	Cardiovascular	Respiratory	Other
Rat*	Dark claws and feet; eyes bulge and pale	Shallow rapid breathing; grunting on expiration	Red starring around eyes and nose; cyanosis, congestion and jaundice in mucous membranes or non-pigmented and non-hairy areas; square fast (dehydration)
Rabbit		As rat	White discharge from eyes, nose, and on inside of fore paws; cyanosis, congestion and jaundice in mucus membranes, or non-pigmented and non-hairy areas
Guinea pig		As rat	Cyanosis, congestion, and jaundice in mucus membranes or non-pigmented and non-hairy areas
Dog		As rat	Salivation and panting. As guinea pig. Raised body temperature; increase in specific gravity of urine and decrease in volume; sweaty paws, pupils dilate, eyes glazed
Cat		As dog	As dog. Circumanal gland discharge: third eyelid may protrude
Monkey		As dog	As dog

* Many signs in rats may also be seen in mice.

Reprinted from Morton, D. B. and P. H. M. Griffiths. 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Record* 116:431-436.

TABLE A-5 Species-specific signs of behavior indicating pain, distress, or discomfort in experimental animals

Species	Posture	Vocalising	Temperament	Locomotion	Other
Rat*	Persistent dormouse posture	Squeals on handling or pressure on affected area	May become more docile or aggressive		Abdominal writhing in mice. Eats bedding; eats neonates
Rabbit	Looks anxious, faces back of cage (hiding posture)	Piercing squeal	Kicks and scratches or dozey		No spillage of food or water; eats neonates
Guinea pig		Urgent repetitive squealing	Rarely vicious; usually quiet; terrified, agitated	Drags legs back	No spillage of food or water
Dog	Anxious glances: seeks cold surfaces; tail between legs; hangdog look	Howls, distinctive bark	Aggression or cringing and extreme submissiveness, runs away	As guinea pig. Raised body temperature; increase in specific gravity of urine and decrease in volume; sweaty paws, pupils dilate, eyes glazed	Penile protrusion; frequent urination
Cat	Tucked-in limbs, hunched head and neck	Distinctive cry or hissing and spitting	Ears flattened; fear of being handled; may cringe		
Monkey	Head Arms across body	Screams	Facial grimace		

* Many signs in rats may also be seen in mice.

Reprinted from Morton, D. B. and P. H. M. Griffiths. 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Record* 116:431-436.

TABLE A-6 Common clinical signs associated with pain in small mammals

Production of fewer, smaller, or no fecal pellets	Reluctance to curl when sleeping (ferrets)
Anorexia	Tucked into abdomen
Half-closed, unfocused eyes	Strained facial expression, bulging eyes
Aggression	Increased frequency and depth of respiration or shallow breathing
Pushing abdomen on the floor	Lameness/ataxia
Stiff movements	Polyuria/polydipsia (especially with GI pain)
Immobility/lethargy/isolation	Head extended and elevated
Overgrooming/lack of grooming	Piloerection
Vocalization (squeal usually fear in rabbits)	Porphyrin secretion
Stretching with back arched	Self-mutilation
Stinting on palpation	Squinting (especially ferrets)
Hunched posture	Absence of normal behavior
Teeth grinding (bruxism)	

Reprinted with permission from Macmillan Publishers Ltd: [Lab Animal] (Mayer 2007), copyright (2007). Mayer J. Use of behavioral analysis to recognize pain in small mammals. *Lab Anim* 36(6):43-48.

On the following page is a score sheet that may be used for behavioral phenotyping in mutant mice. As stated in the report, genetically modified mice may exhibit abnormal behaviors, but those behaviors may be characteristic of the background strain or environmental factors rather than a result of genetic modification. Background strain effects are particularly important where new genetic lines are not completely inbred. In those cases, variation should be expected as a result of different proportions of the progenitor background strains in each animal. Careful review of the characteristics of the background strains is necessary to avoid erroneously attributing differences in test results to the genetic modification. The score sheet was developed by Julie Watson, MA, VetMB, DACLAM, Johns Hopkins University Department of Molecular and Comparative Biology, adapted from Crawley and Paylor (1997).

SHEET A-1 Investigational screen for behavioral phenotyping

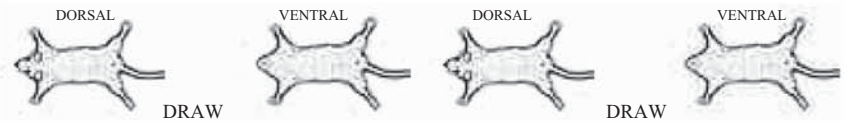
Behavioral Phenotyping Level 1 Screen *Accession #*_____

Date_____Investigator_____Genotype_____

Background strain(s) _____Inbred / N#___ Tg /TM KO/KI/Cond Gene Name _____

Key: 0 = zero; 1 = slow or reduced; ***2 = normal****; 3 = hyper*

Animal #		WT Hemi -/-		Animal #		WT Hemi -/-	
DOB/Age		Sex M F		DOB/Age		Sex M F	
Weight (g)		Fur color		Weight (g)		Fur color	
Condition Score				Condition Score			
Empty Cage 2 mins:		Exploring 0..1..**2**..3		**Empty Cage 2 mins:**		Exploring 0..1..**2**..3	
Gait abnormal	Y N	0= <1 side; 1 =< 1 circuit;		Gait abnormal	Y N	0= <1 side; 1 =< 1 circuit;	
Posture abnormal	Y N	2= multiple circuits; 3= frantic		Posture abnormal	Y N	2= multiple circuits; 3= frantic	
Freezing	Y N			Freezing	Y N		
Wild running	Y N	Digging 0..1..**2**..3		Wild running	Y N	Digging 0..1..**2**..3	
Stereotypies	Y N	Grooming 0..1..**2**..3		Stereotypies	Y N	Grooming 0..1..**2**..3	
Escape	Y N	Rearing 0..1..**2**..3		Escape	Y N	Rearing 0..1..**2**..3	



DRAW DRAW

Bald patches/abnorm alities Bald patches/abnorm alities

Bald patches?	Y.. **N**	Piloerection?	Y..N	Bald patches?	Y.. **N**	Piloerection?	Y..N
Physical abnormality	Y..N	Whisker damage	Y..N	Physical abnormality	Y..N	Whisker damage	Y..N
Body tone	0..1..**2**..3	Whisker response		Body tone	0..1..**2**..3	Whisker response	
		NA	0..1..**2**..3			NA	0..1..**2**..3
Petting escape	0..1..**2**..3	Ear twitch	0..1..**2**..3	Petting escape	0..1..**2**..3	Ear twitch	0..1..**2**..3
Passivity	0..1..2..3	Palpebral reflex	0..1..**2**..3	Passivity	0..1..2..3	Palpebral reflex	0..1..**2**..3
Trunk curl	0..1..**2**..3	Forelimb place	0..1..**2**..3	Trunk curl	0..1..**2**..3	Forelimb place	0 ..1..**2**..3
Righting	0..1..2..3	RL withdraw	0..1..**2**..3	Righting	0..1..2..3	RL withdraw	0..1..**2**..3
Visual placing	0..1..**2**..3	Biting	0..1..2..3	Visual placing	0..1..**2**..3	Biting	0..1..**2**..3
Reach c touch	0..1..2..3 NA	Clicker	0..1..**2**..3	Reach c touch	0..1..2..3	Clicker	0..1..**2**..3
		Grip: **>60**	<60 time _____			Grip: **>60**	<60 time _____

Notes: _____

_____

Adapted from Crawley, J. N. and R. Paylor. 1997. A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Horm Behav* 31:197-211.

The following score sheets have been developed to assess animals in toxicology studies. This assessment is based on a detailed and systematic observation scheme that identifies and scores abnormalities according to a predetermined scale. The recorded symptomatology will determine the diagnosis and subsequent alleviatory actions. They can be adapted to any protocol or animal care facility system as long as the behavioral definitions are uniform across the same facility.

SHEET A-2 Investigational screen for toxicology studies

Step 1.

Daily Cageside Observations

This examination is typically performed with the animals in their cages and is designed to detect significant clinical abnormalities that are clearly visible upon a limited examination and to monitor the general health of the animals. The animals are not hand-held for these observations unless deemed necessary. Significant abnormalities that could be observed include but are not limited to: decreased/increased activity, repetitive behavior, vocalization, incoordination/limping, injury, neuromuscular function (convulsion, fasciculation, tremor, twitches), altered respiration, blue/pale skin and mucous membranes, severe eye injury (rupture), alterations in fecal consistency and fecal/urinary quantity.

Clinical Observations

Study personnel will conduct careful, hand-held, clinical examinations during the live phase of the study. The categorical observations made during this examination use a description to record the severity. These observations can be made at any time during the study.

a. Abnormal behavior: Description of unusual behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance) not noted during the cageside portion of examination.
b. Abnormalities of the eye: Any additional descriptive observations concerning the eye, including, but not limited to, cloudiness, opaqueness, overall size, ruptures, etc.
c. Abnormal urine or feces: Description of animal excreta used to assess general health of animal, includes changes in color or quantity.
d. Abnormalities of the gastrointestinal (GI) tract: Description of atypical visual finding related to the gastrointestinal tract (e.g., prolapsed rectum, decreased water or food intake, reflux of test material).
e. Injury: Description of injury the animal has sustained.
f. Missing extremity: Description of missing body part, includes tail, ears, limbs, etc.
g. Abnormal muscle movements: Description of unusual movements (e.g., tremors or convulsion).
h. Palpable mass/swellings: Description of unusual growths or swellings. Includes the location, onset, appearance, and progression of any finding.
i. Abnormal posture: Description of unusual posture or stance.
j. Abnormalities of the reproductive system: Description of atypical visual findings in the reproductive organs, including but not limited to: prolapsed vagina, unretracted penis, scrotum bluish, enlarged testicles.
k. Abnormal respiration: Description of changes in respiration including shallow, slow, rapid, or mouth breathing.

continued

SHEET A-2 Continued

l. Abnormal skin or hair-coat/mucous membranes: Description of atypical skin or mucous membrane color, changes in hair coat, loss of fur, etc.
m. Excessive soiling: Description and location of increased body soiling.
n. General abnormalities: Description of any other atypical finding not fitting any of the previous observation categories.

Step 2. Detailed Clinical Observations (DCO)

The purpose of the DCO examination is to provide information on the physical health of the animals for the duration of a study, as well as to document any changes in health status that may have occurred in response to chemical treatment of the animals. This examination, scheduled periodically during a study, is conducted in a careful and systematic manner. The examination begins at the head of the animal and works toward the rear of the animal. The observations are ranked according to severity.

A. Cage-side observations.
- Abnormal movements or behaviors: Unusual body movements (e.g., tremors, convulsions), abnormal behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance).
- Resistance to removal: The degree to which the animal attempts to escape capture is scored. The observer will slowly present a gloved hand into the cage and will grasp the animal over the shoulder area or by the tail.
  1 = Decrease—clearly less resistance to capture than typical
  2 = Typical—minimally to actively avoids capture and may be mildly aggressive
  3 = Increase—clearly more resistance to capture than typical and is very aggressive (attempts to bite)

B. Hand-held observations recorded while handling an animal.
1. Ranked observations—the following use a defined scale to rank the degree of severity:
   a. *Eye observations*: Eyes are bilaterally examined; however, if a unilateral observation is made, a concurrent observation is not made for the other eye if it is within typical limits.
   (1) Palpebral closure
   1 = Closed (50% to completely closed)
   2 = Open
   3 = Protruding eyes
   (2) Pupil size (aided by penlight): Under typical examination conditions (white light), the typical appearance of the pupils in albino animals is complete constriction. Therefore a decrease in pupil size cannot be observed.
   0 = Unable to evaluate
   1 = Decrease—clearly decreased pupil size compared to typical
   2 = Typical—completely constricted pupils
   3 = Increase—clearly increased pupil size compared to typical
   (3) Lacrimation (noncolored periocular wetness)
   1 = Decrease—extremely dry appearance of cornea
   2 = Typical—glistening cornea (moderate dryness or wetness)
   3 = Increase—extensive wetness around the eyes

continued

SHEET A-2 Continued

b. *Degree of salivation*:
   1 = Decrease—oral dryness
   2 = Typical—limited to moderate perioral wetness, but lips and chin are dry
   3 = Increase—extensive wetness around the mouth and lips

c. *Muscle tone*: An assessment of muscle tone at the time of the hand-held observations.
   1 = Decrease—clearly less muscle tone than typical
   2 = Typical—animal is neither very relaxed nor very tense
   3 = Increase—clearly more muscle tone than typical

d. *Extensor-thrust response*: Extent of reflex response to brisk pushes (by finger) on the plantar surface of the hind feet.
   1 = Decrease—clearly less response than typical
   2 = Typical—clearly detectable extensor-thrust response
   3 = Increase—clearly more response than typical

e. *Reactivity to stimuli*: The degree to which an animal struggles to get free from hand-held restraint is ranked.
   1 = Decrease—very slight or no struggling
   2 = Typical—mild to moderate struggling, animal may vocalize
   3 = Increase—aggressive escape behavior, may try to bite observer and usually vocalizes

2. Categorical observations—these are described in step 1

C. Open-Field Observations—Ranked observations made by placing the animal on a level surface.

1. Responsiveness to touch: The ventral aspect of the tail is lightly stroked using a finger. Typically, the animal will lift its tail and wrap it around the finger when lightly touched.
   1 = Decrease—does not lift tail, but may briefly hold tail in the air when manually lifted; no response to touch
   2 = Typical—lifts tail when touched
   3 = Increase—lifts tail and acts startled, may turn toward finger in an attack response

2. Gait evaluation: Open-field observations are used for gait evaluation. If the animal remains motionless in the open field, it may be forced to walk on its forelegs while the hindlegs are held off the floor.
   1 = Unable to walk
   2 = Clear knuckling, stumbling and poor coordination, may include falling and/or dragging of one or more limbs
   3 = Typical—smooth and coordinated gait

An alternative example is the following score sheet developed at the University of Birmingham Biomedical Sciences Unit, courtesy of David B. Morton, BVSc, PhD.

SHEET A-3 General screening and applicability

Strain:			**Start weight**				
Mouse ID:			**Date:**				
DAY							
TIME							
Appearance							
Inactive, Less active (L)							
Ataxic							
Stary coat							
Dull eyes							
Huddling							
Isolated							
Pinched face							
Eyes half closed							
Discharge eyes/nose							
Not grooming							
Scratching							
*Abnormal breathing							
**Type of breathing							
Feces pellets soft/hard							
Not eating							
Not drinking							

continued

SHEET A-3 Continued

Hunched posture							
Boarding of abdomen							
Tiptoe walking							
Handling							
Aggressive							
Not inquisitive/alert							
Crusty eyes/nose							
Reluctant to move							
Dehydration/skin tone							
Vocalisation							
Hyperactive							
Bodyweight							
% weight change from start							
% weight change previous day							
Body temperature							
Treatment							
Prolapse							
Overgrown teeth							
Other							
NAD							
Vet/PI contacted							
Signature:							

Scoring details
* Abnormal breathing: Breaths/min
** Type of breathing: R: Rapid, S: Shallow, L: Labored, N: Normal

As stated in Chapter 4, establishing surrogate or humane endpoints as part of the experimental protocol and before experiments commence is one of the ways to minimize and alleviate distress in laboratory animals. The following is an example of a tiered scoring system of defined humane endpoints specifically developed for an arthritis mouse model. In this system the levels range from 0-5. When the arthritic wound is judged to be between levels 0-3, the animals are evaluated weekly by the investigator/veterinarian/animal care team. When the wound advances to level 4, the animals are evaluated daily. All animals whose wounds reach level 5 on any day or that remain at level 4 for ten consecutive days are euthanized. Because more than one person evaluates the animals, some variation among the animal care staff does exist, a fact that should be taken under consideration.

SHEET A-4 Establishing humane or surrogate humane endpoints

Collagen Mouse Scoring Sheet

0 No reaction

1 Small scab +/- Reddening at tail base 

2 Moderate scab +/- Swelling at tail base 

3 Extreme scab +/- Swelling up to one third of tail +/- small or superficial ulcer (ligaments not visible) 

4 Tail ligaments visible

5 Tail ligament visible and one or more signs of poor health below:
→ **Constriction at tail base with swelling/discoloration of most of the tail**
→ **Agitation**
→ **Subdued behaviour**

An animal that scores 4 for 10 consecutive days or a score of 5 on any day is euthanized.

Humane endpoints for the Collagen-induced Arthritis Mouse Model. Developed by Ghislaine Poirier, DVM, PhD, GlaxoSmithKline Pharmaceuticals.

Finally, the Committee acknowledges that to date there is lack of consensus on the best way to achieve "normal species-specific behavior" within the conditions most commonly provided for laboratory animals. To this effect, a *pair testing* record from the Wisconsin National Primate Research Center is included (courtesy of Joseph Kemnitz, PhD), which is used to document the process of social acclimation and housing of nonhuman primates. The animals are paired and their interactions are observed. Primates with undesirable behaviors are identified and appropriate measures are taken.

SHEET A-5 Nonhuman primate pair testing record

Wisconsin National Primate Center
Pair Testing Record

Species	Animal IDs	Date	Project#	Initials

BHAV: Test#: _____ Time In: [ ] Time Out: [ ]

Test Conclusion: Compatible – will pair
Incompatible – will not pair
Inconclusive – another test needed
Successfully paired / Date paired _____

Shared food: Yes ____ No ____

Aggression demonstrated: Yes _____ No _____

If yes, describe below and note which animal performed/initiated aggressive actions. Example: threatening, grabbing, biting, fighting

Affiliation demonstrated: Yes _____ No _____

If yes, describe below. Examples: sitting in proximity/near each other, sitting in contact/together, grooming, mounting

Comments:

_____
_____
_____
_____
_____

_____
Initials

About the Authors

Peter A. Ward *(Chair)*, MD, IOM, Professor, Department of Pathology, School of Medicine, University of Michigan. He has extensive experience with laboratory animal welfare issues through his past membership and chairmanship of ILAR Council. He served as Chair of the ILAR Committee to Update Science, Medicine, and Animals, as well as Chair of the ILAR Committee on Methods for Producing Monoclonal Antibodies. He also has an extensive background in immunopathology, inflammation, and mechanisms of antibody formation.

Robert J. Blanchard, PhD, is Professor of Psychology at the University of Hawaii. He has extensive experience managing animals in environments that model chronic social stress, fear, aggression, and defense. His research is focused on pharmacological and neural control of natural behavior, animal models of emotion, and ethoexperimental analysis of aggression and defense. He is a past president of the International Behavioral Neuroscience Society and the International Society for Research on Aggression. Dr. Blanchard has also served on the editorial boards for *Neuroscience and Biobehavioral Reviews, Journal of Comparative Psychology,* and *Pharmacology, Biochemistry & Behavior.*

Valerie Bolivar, PhD, is a Principal Investigator and Director of the Mouse Behavioral Phenotype Analysis Core at Wadsworth Center, New York State Department of Health and Assistant Professor, Department of Biomedical Sciences at the State University of New York School of Public Health. Her research is on the behavioral differences in rodents associated with different

genetic backgrounds and disease states. She is particularly interested in the role of genetics on complex behavioral phenotypes and has been working with mouse models of human disease for over 16 years. She has previously served as a member of the research ethics committee at St. Mary's University, Nova Scotia, Canada.

Marilyn J. Brown, DVM, is Executive Director of Animal Welfare and Training at Charles River Laboratories. She is a Diplomate of the American College of Laboratory Animal Medicine. She has experience dealing with issues pertaining to animal welfare, as well as serving on a number of NRC committees, including the ILAR Committee to Revise the Guide for the Care and Use of Laboratory Animals (1996). She also served as a reviewer for the 1992 ILAR report *Recognition and Alleviation of Pain and Distress in Laboratory Animals*. Dr. Brown has been the recipient of numerous honors and awards, including being awarded the title of De Facto Diplomate for the European College of Laboratory Animal Medicine.

Fon Chang, DVM, is Senior Staff Veterinarian at AstraZeneca Pharmaceuticals LP. He completed residencies in Laboratory Animal Medicine and in Animal Behavior at UC-Davis. His experience on animal welfare and behavior issues includes behavioral management of research animals and behavioral phenotyping of genetically engineered mice. Dr. Chang's professional affiliations include the American Association for Laboratory Animal Science, the American Society of Laboratory Animal Practitioners, and the American Veterinary Medical Association.

James P. Herman, PhD, is Professor of Psychiatry and Director of the Laboratory of Stress Neurobiology at the University of Cincinnati. His research pertains to rodents and the structural and functional neurocircuitry involved in the stress response, molecular effects of stress in the brain, and gender differences in the stress response. Dr. Herman was recently a panel member of the American Physiological Society Workshop on Managing Pain and Distress in Experimental Animals, and has been an invited speaker on the topic of distress for the AALAS and PRIM&R/ARENA national meetings.

Robert Hubrecht, PhD, has been with the Universities Federation for Animal Welfare (UFAW) since 1992, where he has served as Deputy Director since 1996 (UFAW is a unique scientific and technical animal welfare organization in the UK, which uses scientific knowledge to improve the welfare of animals kept as pets, in zoos and laboratories, and on farms). He has a long career in the fields of animal welfare and ethology. This includes, in addition to a number of academic positions, service as a member of the Animal Procedures Committee (APC). The APC advises the UK Home

Secretary on matters relating to the Animals (Scientific Procedures) Act 1986, which regulates the use of animals in research in the UK. He has also served as a chair or member of several subcommittees of the APC, including the Housing and Husbandry Subcommittee, the Primates Subcommittee, the Severity Working Group, and the Working Group on the Use of Marmosets. Dr. Hubrecht has published numerous articles on the subject of animal behavior. He was also a recipient of the GlaxoSmithKline Animal Welfare Prize in 2004.

David M. Lawson, PhD, is Professor of Physiology at Wayne State University School of Medicine, Detroit. He has extensive knowledge of laboratory animal physiology and the effect of husbandry and enrichment on physiology. His research focuses on integrative responses of experimental rodents to common experimental manipulations and housing conditions. Dr. Lawson is a member of the American Physiological Society, The Endocrine Society, and the American Association of Laboratory Animal Science. During the period 1978-2006, he served for 21 years as member or chair of the University's institutional animal care and use committee.

Steven F. Maier, PhD, is a University of Colorado Distinguished Professor and the Director of the Center for Neuroscience. His research is focused on maladaptive behaviors, analgesia, and the effect of stress on immune function and neurochemistry. Dr. Maier codeveloped the first animal model of learned helplessness and devised ways to alleviate this type of distress. He has received numerous awards including the Norman Cousins and the Neal Miller Distinguished Lectureships, and is a Fellow of the American Psychological Association, the American Physiological Association, and APA, APS, and AAAS. He has been on the editorial boards of numerous journals and authored or coauthored over 250 scientific papers and 300 abstracts.

David Morton, PhD, is professor emeritus of biomedical science and ethics at the University of Birmingham in the UK and a laboratory animal veterinarian. His research focuses on the recognition of adverse states, such as distress in animals, their assessment and alleviation, their practical use in animal research such as in the development of humane endpoints, and their impact on science and the animals. He is a member of the European Food Standards Authorities Scientific Panel on Animal Health and Welfare, the UK's Farm Animal Welfare Council, the Companion Animal Welfare Council, sits on various human and animal ethics committees, and has received several national and international awards for his work on the Three Rs. He has published more than 200 papers and chapters, and recently chaired European committees on primate and rabbit welfare, and various aspects of animal research for the revision of the EU Directive.

Steven M. Niemi, DVM, is Director of the Center for Comparative Medicine at Massachusetts General Hospital and an Instructor of Pathology at Harvard Medical School. Dr. Niemi is a Diplomate of the American College of Laboratory Animal Medicine and served on its Board of Directors. He is an Ad hoc Consultant and Specialist with AAALAC International. He is a past President of the Scientists Center for Animal Welfare and a member of the American Association for Laboratory Animal Science, American Veterinary Medical Association, and Veterinary Cancer Society. Dr. Niemi was selected because of his experience in refinement and reduction in animal testing, alternatives to animal experimentation, and use of nontraditional laboratory animals, as well as his unique perspectives from his professional experience with both academic and industrial research institutions.

Melinda Novak, PhD, is Professor and Chair of the Department of Psychology at the University of Massachusetts in Amherst. Her research examines the psychopathology of monkeys, with an emphasis on self-injurious behavior, and the effects of early experience on cognitive and social development in rhesus monkeys. She has previously served as President of the American Society of Primatologists, as well as chair or member of a number of committees, including the USDA Animal and Plant Health Inspection Service's Advisory Panel on the Psychological Well-being of Captive Primates, the ILAR Committee on Psychological Well-being of Captive Primates, and the Board of Trustees of AAALAC International.

Stephen L. Zawistowski, PhD, is Senior Vice President and Science Advisor of the American Society for the Prevention of Cruelty to Animals. He has worked extensively in animal behavior and welfare. Dr. Zawistowski is a Certified Applied Animal Behaviorist and is the Chairman of the Animal Behavior Society's Board of Professional Certification and a Certified Technical Animal Rescue Specialist as well. He has been a member of several animal protectionist organizations and committees, including the Scientific Advisory Panel for the World Society for the Protection of Animals (WSPA), the Scientific Advisory Committee for Humane Farm Animal Care, and the National Council on Pet Population Study and Policy. He is a founding co-editor of the *Journal of Applied Animal Welfare Science* and served on the BANR/ILAR committee that reviewed the Smithsonian's National Zoological Park.

Index

B

C

D

S

T

U

V

W