Guidelines for the Use of Fishes in Research (2004)

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Aaron Lerner, Publications Manager
American Fisheries Society
5410 Grosvenor Lane
Bethesda, MD 20814

phone: 301-897-8616, ext. 231
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Guidelines for the Use of Fishes in Research

American Fisheries Society

American Institute of Fishery Research Biologists

American Society of Ichthyologists and Herpetologists

Use of Fishes in Research Committee members:

J. G. Nickum, Chair, H. L. Bart, Jr, P. R. Bowser, I. E. Greer, C. Hubbs, J. A. Jenkins, J. R. MacMillan, J. W. Rachlin, J. D. Rose, P. W. Sorensen, and J. R. Tomasso

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Acknowledgments

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Thank you.

John G. Nickum, Chair, UFR Committee

Preface

The American Fisheries Society, the American Society of Ichthyologists and Herpetologists, and the American Institute of Fishery Research Biologists are

professional societies that are focused on scientific understanding and conservation of fish and fisheries. These professional organizations are associations of scientists and resource managers whose primary interests are fish and fisheries. Their policies and position statements are based primarily on information that has been developed through scientific processes, but they also reflect ethical concerns, including the conservation of the diversity and number of fishes and respect for life and life processes in all forms. Research investigations of fishes, the environments in which fishes are found, the factors influencing the health and well-being of fishes, and the variety of human activities that depend upon and/or affect fishes are core activities for all three societies. These societies, however, believe that their members are responsible not only for advancing scientific knowledge and understanding of fish and fisheries but for improving human appreciation for these animals and the industries that they support. All three societies actively promote research and the dissemination of information derived from that research. They also advocate respect for life processes, the forms of life that make up the various ecosystems, and the humane treatment of animals used in research investigation.

The understanding and welfare of animals used in research can be served best by using a multidisciplinary approach in which data and expertise from several scientific disciplines, including such areas as ecology, behavior, nutrition, genetics, physiology, anatomy, and fish health, are merged in order to address issues concerning animal care and use. At the same time, it must be understood that research is conducted in a variety of human culture settings. Ideally, scientific procedures, methods of analyses, interpretations of statistically valid data, and conclusions based on scientific studies should be consistent across all cultures, even though personal belief systems can and do influence concepts as to what practices and methods are, or are not, consistent with humane treatment of animals. The members of the Uses of Fishes in Research (UFR) Committee who developed the new and revised Guidelines for the Use of Fishes in Research (referred to as Guidelines in this document) are scientists, and each member carries a deep respect for life processes and the myriad forms of life. The Guidelines that follow reflect not only the scientific expertise of the UFR Committee members but also express the desire of the committee members to promote scientifically valid research on fish and fish habitats, research that is conducted in a manner acceptable to the societies within which the research takes place and to the benefit of the fishes and the ecosystems in which they live.

The new Guidelines have been developed to replace the Guidelines for the Use of Fishes in Field Research (ASIH et al. 1987, 1988) and to expand the coverage to include laboratory research. The new Guidelines include listings of web sites that should be of value; however, readers are cautioned to check such sites frequently because their content or addresses may change. If readers experience difficulty in reaching a specific site, they are advised to access the general site and then search for specific references.

Statement of Purpose

The new and revised Guidelines were developed to provide a structure that ensures appropriate attention to valid experimental design and procedures while also ensuring humane treatment of the experimental subjects. At a practical level, the Guidelines are intended to provide general recommendations on field and laboratory activities, such as sampling, holding, and handling fishes; information on administrative matters, including regulations and permits; and advice concerning ethical questions, such as perceptions of pain or discomfort that may be experienced by experimental subjects. These Guidelines must be recognized as *guidelines*. They are not intended to provide detailed instructions but rather to alert researchers to a broad array of topics and concerns with which they should become familiar before they initiate studies. Also, the Guidelines were not designed for the myriad fish handling activities conducted by fisheries managers nor for aquaculture operations or commercial fishing. However, the principles upon which these Guidelines are based are broadly applicable, and many of the recommended practices can be adapted to fishery management situations.

Understanding the differences between fish and other vertebrate animals, especially mammals, is critically important to the conduct of scientifically valid research on fishes. The UFR Committee emphasizes that: (1) mortality patterns among fishes differ greatly from those of mammals, especially in the fact that thousands or tens of thousands of eggs, or even early life stages, may produce only a few adult animals; (2) because of these mortality patterns, research on fishes, especially field research or research on early life stages, normally requires much larger numbers of research subjects than does research on mammals; and (3) the handling requirements for fishes are fundamentally different from the requirements for mammals and other vertebrate animals in general. Policies, regulations, and recommendations developed for research on mammals, birds, reptiles, or even amphibians are frequently inappropriate for research on fishes. These Guidelines provide recommendations that address the ethical concerns that underlie guidelines for other vertebrates while recognizing the unique nature of fishes.

These Guidelines have been developed for general use by researchers within the United States; therefore, the roles, responsibilities, and information needs of Institutional Animal Care and Use Committees (IACUCs) are given specific attention. Researchers in nations other than the United States should disregard specific references to state and federal laws and regulations and may not have internal committees similar to IACUCs. We suggest, however, that the principles described in these Guidelines are applicable to research on fishes everywhere. Researchers in other nations can modify the specific provisions pertaining to the United States and adopt guidelines consistent with the laws and regulations of their own government.

The UFR Committee suggests that these Guidelines should be endorsed and adopted (adapted, where necessary) by those state and federal agencies with regulatory responsibilities for fishes as well as by universities and research institutions.

I. Introduction

Experimental studies using live, intact creatures have played, and continue to play, an essential role in developing new knowledge and better understanding of life processes, life forms, and the environment in which these forms and processes occur. The enormous evolutionary radiation of fishes comprises at least 25,000 species. Fishes exist in myriad forms and have developed many unique physiological, behavioral, and ecological specializations. Fishes occupy a variety of niches in virtually every kind of aquatic habitat. Understanding their biology simply cannot be accomplished in the absence of experimentation with live, intact animals.

Among the reasons for studying fishes are the following: fishes are useful indicators of environmental quality and ecological integrity; fishes provide an important source of food for many of the world's humans; catching and observing fishes are very popular and economically important recreational and commercial activities for millions of people; the unique adaptations and physiological specializations of fishes make them especially suitable for use as physiological and biomedical models; human existence is dependent on understanding our place and functions in the world's ecosystems, an understanding that cannot be accomplished without accurate and detailed knowledge of the biology of fishes.

The use of animals in research carries with it responsibilities for efficient, effective design of experimental studies and for humane treatment of the experimental subjects (Klontz and Smith 1968; Snieszko 1974; DeTolla et al. 1995; Klontz 1995). Animals experiencing physiological trauma may exhibit abnormal behavioral or physiological responses that could defeat the purposes of the investigation.

The diversity demonstrated by the over 25,000 species of fishes creates many opportunities for new research but also makes the task of developing specific protocols that apply to all species and all circumstances impossible. Instead, broad guidelines building on the most current, scientifically valid information are provided in these Guidelines for interpretation and application by the investigator, who frequently will be "the authority" on the species or system under study. Ultimate responsibility for the ethical and scientific validity of each study and the methods employed must rest with the investigator. However, government agencies, reflecting the beliefs and values of the citizenry and acting on their behalf, now demand that researchers follow codes prescribing acceptable strategies, techniques, facilities, conditions, and post-experimental disposition of animals used in research.

Some individuals have argued that fishes may not be included under laws and policies aimed primarily at mammals and birds; however, the Health Research Extension Act of 1985 (Public Law 99-158, 20 November 1985) included fishes within its jurisdiction and

responsibilities. Fishes are specifically included within the scope of the Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences (ILARC; ILARC 1996). None of the laws, nor general guides such as the Guide for the Care and Use of Laboratory Animals, provide detailed guidance; therefore, additional supplemental guidelines are needed. In fact, the ILARC Guide specifically calls for the development of detailed guidelines by knowledgeable groups. Generally, scientific societies with expertise concerning the individual classes of vertebrate animal are considered to be the most appropriate sources for the supplemental information needed to implement existing policies. The Guidelines for the Use of Fishes in Field Research were developed and jointly published by the American Society of Ichthyologists and Herpetologists (ASIH), the American Fisheries Society (AFS), and the American Institute of Fishery Research Biologists (AIFRB; ASIH et al. 1987, 1988). The 1987–1988 guidelines emphasized field research and did not discuss laboratory research because they were developed in response to a change at that time that specifically included field research under federal rules. The new and revised Guidelines (herein) expand the coverage of the 1987–1988 guidelines to include laboratory studies as well as field studies. The revised Guidelines incorporate new findings and understandings that have developed since 1988 as well as a convenient checklist to assist researchers preparing Institutional Animal Care and Use Committee (IACUC) applications (see Appendix).

The Role of Institutional Animal Care and Use Committees

The IACUCs are appointed by the chief executive officer of each institution. The IACUCs have certain federally mandated responsibilities, such as review of protocols and periodic evaluations of the program of animal care and use, including inspections of facilities.

Typically, each institution develops its own procedures following federal guidelines to address the basic responsibilities of its IACUC. The IACUC conducts semiannual program evaluations, inspects animal facilities, reviews protocols, maintains IACUC records, and develops annual reports for the responsible institutional or federal official. The IACUC is also responsible for ensuring that adequate veterinary care is provided, that a human occupational health and safety program is part of the overall animal care and use program, and that animal facilities, including husbandry of animals, are properly managed. The membership of the IACUC varies from institution to institution but normally includes five or more individuals. A veterinarian, a practicing research scientist, an individual whose primary concerns are in a nonscientific area, and an individual who is not affiliated with the institution other than as a member of the IACUC typically are included.

The IACUC has a mandate to investigate and evaluate concerns regarding the care and use of animals at the institution. Concerns may be raised by staff or employees of the institution, individuals in the community, or even members of the IACUC. The IACUC is

empowered to suspend a project if it finds violations of the Public Health Service (PHS) Policy and Government Principles Regarding the Care and Use of Animals (ILARC 1996, Appendix D). For further information on the role and responsibilities of IACUCs, see APHIS (1992), Thomas and Greene (1994), ILARC (1996), or Silverman et al. (2000; also see Public Law 99-158).

II. General Considerations

Certain general considerations apply to nearly all research investigations on fishes, whether conducted in the field or in a laboratory setting. This section proposes materials and procedures that can be adapted to the situation and circumstances of each researcher.

Research studies should have well-understood and justifiable objectives that address, within the context of the research discipline, basic needs for knowledge and understanding the world in which we live. The quality of research is affected by many factors, beginning with the researcher's ability to ask questions that can be answered by scientific methods, establish hypotheses that can be tested, and develop and publish a set of research procedures and results that can be repeated and verified by other scientists. The validity of research results is affected by the experimental design, the analytical procedures employed, and the quality, including health status, of the fish used in the studies. Descriptions of the experimental design, methods, and procedures are essential because they must be known in order to facilitate independent repetition and verification of scientific observations and conclusions. The quality and appropriateness of the fishes used, both the species and the individuals, can seriously influence the results and conclusions, thereby having dramatic effects on the number of animals needed and the number of times that the study must be repeated. These effects, in turn, will have important animal welfare and financial implications. Research scientists have long recognized the importance of animal welfare considerations; however, formal guidelines for the use of fishes in research were not common in the United States before 1985, when requirements that research proposals obtain the approval of an IACUC were imposed. Although the principles and procedures described in these Guidelines have been designed to address requirements imposed by IACUCs in the United States, the general concepts should be applicable to researchers in all situations and all countries.

Approval of Research Plans by IACUCs

When approval by an IACUC is required, investigators must prepare written statements or applications for animal use that assure that all applications, proposals, and actual research will meet certain basic requirements. Such assurances include limiting unneeded replications of research, use of appropriate species, and adequate experimental design. Investigators must be familiar with the species to be studied, or closely related surrogates, so as to be able to provide environmental conditions essential for the well-being of the

experimental subjects and to be able to recognize their responses to disturbances, including capture and restraint, or other changes in environmental conditions that may be applicable to the particular study. Copies of IACUC plans are maintained by the IACUC and the individual researcher.

Quality Assurance Plans and Standard Operating Procedures

Quality assurance plans (QAPs) and standard operating procedures (SOPs) are not required by many universities or nongovernmental institutions; nevertheless, the concepts upon which these documents are based are recommended as useful tools for maintaining overall research quality.

The QAPs and SOPs usually are required if research data and conclusions will be submitted to certain regulatory agencies, such as the Food and Drug Administration (FDA) or the U.S. Environmental Protection Agency (USEPA). Specific benefits that can result from the establishment and use of QAPs and SOPs include the following: consistency in repeated procedures, limiting unneeded replications, obtaining the most information possible from the fewest fish and staff, and developing sets of data that can withstand legal challenges.

The QAP is a written document that describes the principles, policies, organizational responsibilities, objectives, implementation actions, and accountability procedures that will ensure appropriate quality throughout a research project. The QAPs document management procedures and ensure that the data collected will qualify for meeting study objectives. The QAPs should be detailed enough to provide clear descriptions of every aspect of the project. They facilitate communications and help to keep projects on schedule and within budget. An important matter that is overlooked frequently in QAPs is tracking and limiting access to research facilities and animals. The quality of research, and even the welfare of experimental animals, can be compromised if access is not controlled and tracked. Posting signs and providing sign-in and sign-out sheets help to ensure that other provisions in the QAPs are followed.

Typically, QAPs are prepared by the principal investigator and are subject to approval by that individual's supervisor(s) and the IACUC. Copies of QAPs should be kept in designated, readily accessible locations in the office of the principal investigator and the department or section where the study will be conducted.

Standard operating procedures document routine or repetitive technical activities, ensuring that work is done correctly the first time, thereby reducing unnecessary repetition and costs. Examples of specific technical tasks that may be used to conduct research with fishes include the following: blood sampling, vaccination protocols, procedures for electrofishing, or techniques for collecting meristic data. The SOPs promote consistency in quality and integrity of data and are useful for maintaining consistency when there are changes in personnel. They also can form an essential part of

effective training programs. General SOPs, not specific to individual studies, may be established as basic procedures for entire research institutions or laboratories. Additional information on SOPs and QAPs is available from regulatory agencies such as USEPA or FDA and from various manuals or texts that address quality assurance.

Statistical Design and Experimental Endpoints

Statistical Design

The number of animal subjects required for an investigation will depend on the questions being explored. Field studies and laboratory studies typically require greatly different statistical designs, with field studies typically requiring much larger numbers. The life stage of the fish used in each study will also affect the numbers needed. Studies of early life stages typically require very large numbers of individuals. In all cases, studies should be designed to use the fewest animals necessary to reliably answer the questions posed.

The use of adequate numbers to establish variance and to assure reliability is essential so as to prevent needless repetition of the study (ASIH et al. 1987, 1988). A true "replicate" is the smallest experimental unit to which a treatment can be applied independently. Pseudoreplication can result from wrongly treating multiple samples from one experimental unit as multiple experimental units or from using experimental units that are not statistically independent (Heffner et al. 1996). Study objectives should be presented as clearly stated hypotheses, and explanations should be provided as to the need for the type and quantity of data to be collected as well as what will constitute an end to the experiment. Power analysis procedures have been useful to many researchers to determine the appropriate number of fish needed to accomplish acceptable, statistically valid results. Researchers are encouraged to consult with a statistician to develop study designs that have the appropriate statistical power to accomplish research objectives.

Mortality as an Experimental Endpoint

In general, experimental endpoints other than death of the experimental subjects should be developed unless death is required by the study protocol. The use of mortality as an endpoint is appropriate when one or both of the following criteria are met:

> (1) Little or no information pertaining to research objectives is available on the species of interest or the experimental variable being imposed. (For example, short-term, limited mortality studies may be used to develop experimental limits for subsequent sublethal studies.)
>
> (2) Mortality data are required or at least used frequently by a sponsoring agency to provide a basis for criteria development as part of a regulatory process. Many studies concerning the effects of pathogens and parasites or studies concerning the effects of drugs and other chemicals require mortality endpoints.

Fish Health Management: Control of Pathogens and Parasites

Healthy fish are prerequisites for reliable data (Jenkins 2000a). Fish utilized in research must be free of any notable microbial presence that could indicate a diseased condition, unless an infectious disease is part of the experimental protocol. Specific pathogen-free fish generally will be satisfactory; however, an unrecognized disease condition, even at chronic or nonlethal levels, can seriously confound research results. If a disease condition is part of the experimental design, the potential effects of the pathogen or parasite on research results should be predictable or constitute a variable that is being tested through the research. If fish are treated for a disease with a therapeutic compound before initiation of experimental studies, they should not be used in such studies until sufficient time has passed to eliminate any residues of the treatment. Consideration of any other effects of the treatment on the representative status of the subject fish must be included in the design of the study and the analyses of data derived from that study.

In both captive-reared and wild-caught fish, one may expect to find various infectious organisms. The presence of such infectious organisms may not cause disease or prevent the use of the host fish in research, but the relative importance of their presence must be evaluated for possible effects on research results (Winton 2001). Testing for specific pathogens or parasites may be warranted. Diagnostic procedures continually improve and allow for greater confidence that pathogens of concern are not present or not present in numbers great enough to affect the accuracy or reliability of research results. It probably is unrealistic to consider fish to be "disease free" in all but certain special cases of captive-bred species. Steps should be taken in consultation with a fish health specialist or veterinarian to address the fish health issues in a manner that provides for the health and well-being of the fish and also supports the research.

If experimental fish are to be treated for a disease, FDA-approved drugs should be used and current FDA regulations followed (Code of Federal Regulations; 21 CFR, part 511.1 [a]), although considerable flexibility is provided by the FDA for research conducted in laboratory settings. In addition, veterinarians are allowed to prescribe extra-label uses of drugs under some circumstances. Institutional, local, or state guidelines pertaining to the administration of drugs must be followed, and USEPA, state, and local regulations pertaining to effluent discharges that may contain drugs must also be observed. The UFR Committee recognizes the fact that many drugs and disease treatments have been used in the past with some degree of apparent success in combating the signs of disease; however, we believe that considerable caution should be exercised in the use of any drug that has not received FDA approval. A list of the substances that seem effective but have not been approved will not be provided in these Guidelines because of the danger that such an inclusion could be considered as endorsement of unapproved drugs. Fish treated with substances that have not been approved by FDA must not be released nor consumed. Complete records of all disease treatments must be maintained because FDA inspectors may order reviews of such files. Approved therapeutic compounds have a label (permission) that provides guidance for the use of that substance with fish. Research

designed to study the efficacy, safety to fish, human safety, or environmental safety of the disease treatments should be designed in consultation with the FDA Center for Veterinary Medicine and approved in advance by FDA. If the fish may eventually be released or if they could become a food item for human consumption, it is imperative that FDA regulations be observed in detail. Additional information may be obtained from the web sites for the FDA and USEPA.

III. Statutory Requirements and Regulatory Bodies

The investigator must have knowledge of all regulations pertaining to the animals under study and must obtain all permits necessary for carrying out proposed studies (ASIH et al. 1987, 1988). Responsibility for compliance rests with the institution and, ultimately, with the principal investigator.

International Regulations and Guidelines

Researchers working outside of the United States should ensure that they comply with all wildlife regulations of the country in which the research is being performed. Work with many species is regulated by the provisions of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Estes and Sessions 1983).

In the world economy, international trade in animals and animal products necessitates regulations designed to prevent the spread of transmissible diseases to individual animals, between groups of animals, and to humans (Jenkins 2000b). Disease risks should be assessed and precautions taken to minimize risks before wildlife is translocated (Cunningham 1996).

The Office International des Epizooties (OIE), created in 1924 and headquartered in Paris, has been a leader in defining international health standards for animals. Initial efforts focused on terrestrial animals, but aquatic animals are included now. Reports from each country are compiled and all governments are informed of the occurrence of disease agents and the course of animal diseases as well as ways to control them. Coordinated studies are devoted to surveillance and control of pathogens and parasites determined to have especially harmful effects. The OIE harmonizes regulations for trade in animals and aquaculture products for nearly 150 member countries. Special commissions of the OIE deal with specific issues such as fish diseases (Fish Disease Code Commission), or general regulatory issues (International Animal Health Code Commission.) Each country's Chief Veterinary Officer is its delegate to the OIE; however, specialists in the specific issues covered by individual commissions are chosen for their expertise rather than agency affiliation.

The Organization for Economic Cooperation and Development has been concerned

primarily with traditional toxicological test methods for human health and eco-toxicology but does provide background information concerning drafting and revising toxicity test methods with fishes. Investigators conducting studies that involve toxicological tests, especially if alternative test methods are being developed or the results will be submitted for regulatory acceptance, should refer to the following National Institutes of Health and Office for Human Research Protections web sites to confirm current standards and check for additional information.

Federal, State, and Local Regulations

In the United States, federal authority for the use of animals in research is found primarily in two agencies, the Department of Health and Human Services (HHS) and the U.S. Department of Agriculture (USDA). If endangered species are involved, the U.S. Department of the Interior or the U.S. Department of Commerce have additional authorities. Authority for each department is found in specific acts of Congress. Legislative mandate for the Public Health Service (PHS/HHS) policy for use of animals in research is provided in the Health Research Extension Act of 1985. The Public Health Research Extension Act charged the Secretary of Health and Human Services with the responsibility to establish guidelines for proper care and treatment of animals used in research and for organization and operation of animal care committees. This act extends beyond research conducted within PHS facilities to all PHS-supported activities involving animals. Within the act, "animal" is defined as "any live vertebrate animal used or intended for use in research, training, experimentation, or biological testing or for related purposes."

The legislative mandate for animal welfare within the USDA is contained in the Animal Welfare Act, as amended (7 USC, 2131–2156). The complete act, including all amendments (1970, 1976, 1985, and 1990) following the 1966 enactment, can be found in United States Code, Title 7, Sections 2131 to 2156. (The USDA regulations implementing the Animal Welfare Act can be found in the Code of Federal Regulations, Title 9.) The Animal Welfare Act authorized the Secretary of Agriculture to regulate transport, sale, and handling of dogs, cats, nonhuman primates, guinea pigs, hamsters, and rabbits intended to be used in research or "for other purposes." The Animal Welfare Act of 1970 (Public Law 91-579, 24 December 1970) expanded the list of animals covered by the act to include all warm-blooded animals determined by the Secretary of Agriculture as being used or intended for use in experimentation or exhibition except horses not used in research and farm animals used in food and fiber research. Although fishes are not included under the Animal Welfare Act, our committee recommends that researchers be familiar with the general content and intent of the act. The Food Security Act of 1985, subtitle F—Animal Welfare (Public Law 99-198, 23 December 1985), also called the Improved Standards for Laboratory Animals Act, clarifies what is meant by "humane care" by mentioning specifics such as sanitation, housing, and ventilation. It specifies that "pain and distress" (see Section IV) must be minimized in experimental procedures and that alternatives to such procedures be considered by the principal

investigator. The establishment of the IACUC is introduced with a description of its roles, composition, and responsibilities to the USDA Animal and Plant Health Inspection Service (APHIS). A thorough compilation of information sources related to fish welfare is available from the USDA (Erickson 2003). Current information on federal regulations may be found on the Public Health Service web site and USDA Animal Welfare Information Center web site.

States may have specific legislative statutes that empower them to regulate the use of animals in research. Typically, these regulations may be found in the laws pertaining to natural resources, health, and agricultural use of fishes and wildlife. Interstate transport of fishes, and in some situations intrastate transport, is usually regulated at the state level. Researchers are urged to determine which state laws may affect the conduct of their research. One source of information is the Legal Information Institute web site, "State Statutes on the Internet".

Local authorities rarely oversee the conduct of research; however, investigators should recognize that local regulations relative to the conduct of their studies may exist. Responsibility for knowledge of these regulations rests with the investigator.

Import–Export Permits: Health Certificates

In addition to government regulations pertaining to the conduct of research, permission usually is required for the transport of animals across state or international boundaries. Currently in the United States, authority for interstate transport of fishes is usually under the jurisdiction of the fish and game agency of the state into which the animals will be transported, while international shipments most commonly are under the control of the U.S. Fish and Wildlife Service (USFWS). The intent of these regulations is to prevent the introduction of exotic disease agents as well as to address concerns associated with endangered or threatened species of animals. The researcher should consult with the appropriate state and federal agencies as well as refer to the provisions of the CITES for further guidance.

IV. Animal Welfare Considerations: Stress and "Pain"

General Considerations and Ethical Concerns

Research involving living animals, including fishes, must be based on experimental designs that can lead to scientifically valid results while also taking the welfare of the test animals into consideration. These standards are commonly accepted because of ethical concerns and because only data derived from healthy animals behaving in "normal" fashions can be considered representative of "normal" biological function. Researchers need to take great care to avoid inducing stress in experimental subjects (especially on a

prolonged basis) because it can evoke physiological and behavioral changes (Barton and Iwama 1991). Additionally, care must be taken to avoid subjecting fish to physiologically damaging (nociceptive) stimuli because they too can evoke stress or aberrations in normal physiological as well as behavioral state. Accordingly, unless the experimental objectives require actions or conditions designed to test responses to stress or nociceptive stimuli, fish should be maintained, handled, and tested under conditions that will not create such responses.

In addition to scientific considerations, ethical standards mandate a respect for all life forms and processes. However, ethical beliefs are based on human value systems, which are both diverse and are not always based on scientific information and methods. While the authors of these Guidelines respect these individual belief systems, this document addresses the conduct of scientific research and, therefore, focuses on established scientific fact and the processes through which such knowledge is developed. We recommend that research organizations develop institutional guidelines that are based on the best scientific evidence currently available concerning the biological nature of fishes. Research plans submitted to IACUCs should address animal welfare considerations as well as provide details of research goals, objectives, and procedures. The extent to which IACUCs incorporate ethical beliefs and personal values concerning animal welfare into their institutional guidelines must be determined within each institution; however, institutions should recognize that imposition of regulations based on personal beliefs and values may compromise the purpose, design, and scientific value of the research.

Stress

Stated in general terms, the scientific study of what is "normal" and "healthy" for organisms, how they achieve their physiological balance, and consequences that result when they do not, is known as the study of stress. This field has focused on how animals have evolved physiological and behavioral mechanisms that meet the challenges of the changing environmental conditions that they typically encounter and that permit them to maintain homeostasis, or self-sustaining balance. The set of environmental variables (conditions) best suited for the well-being of each species encompasses a specific range for each factor and species. Accordingly, when fish are found or maintained within these ranges, a state of homeostatic balance will be expected. Deviations from homeostasis characterize a stress response. While many definitions for stress have been proposed, we employ the definition of Schreck et al. (2001): "a physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of insult." When stressed, fish generally assume a different mode of operating known as "allostasis" (Sterling and Eyer 1988). While allostasis can be adaptive in terms of keeping the animal alive in the face of a stressor, it is almost always maladaptive over the long term when one considers life functions such as growth, reproduction, and immunological health. Accordingly, researchers need to understand factors that might cause stress in their experimental animal(s), the potential consequences of stress responses, and how stress might be avoided through optimization of experimental

conditions.

Minimizing actions and conditions that provoke stress responses also provides the benefit of avoiding behavioral responses, such as escape or aggressive behaviors, that are often interpreted by human observers as manifestations of "discomfort," "distress," or "pain." A characteristic of all "high-quality" guidelines for fish care and health management is emphasis on the importance of avoiding stress or at least minimizing it. Although these Guidelines emphasize the importance of avoiding stress as a confounding factor, we recognize that avoiding it completely is sometimes impossible. However, extensive practical experience in conducting experimental investigations has demonstrated that the stressors involved with routine handling do not compromise research results nor produce pathological effects. The relative health of fishes can be determined without further testing through careful observation of such basic functions as swimming and feeding.

Each investigator and the IACUC are responsible for knowing and understanding the conditions that minimize stress for the species in question. However, extrapolation between taxa must be avoided inasmuch as differences exist between species. The factors and range of conditions appropriate for fishes typically will deviate substantially from those used for mammals. Assumptions and perceptions based on experiences with mammals, especially primates, must not be extrapolated to fishes; however, researchers should be aware of agency policies (i.e., USDA Policy 11) which are based on human pain experiences.

Stages of Stress

It is important to recognize and understand the stages of stress, of which three are generally identified. All three stages warrant specific consideration in the design of animal care protocols. Primary stress responses vary greatly among species and situations but, if not prolonged, usually have few, if any, adverse effects on a fish's physiological well-being. Primary stress is often demonstrated by altered behavior, such as elevated gill ventilation and changes in swimming behavior, perhaps including escape behaviors. A primary stress response is characterized by immediate neuroendocrine responses, such as catecholamine and corticosteroid release, and can be quantified by measuring blood hormones. The secondary stage of a stress response is characterized by changes in blood and tissue function that often are triggered by the primary response. Secondary stress typically occurs within 30 minutes of the primary response and is characterized by a variety of symptoms including increased blood glucose and heart rate, diuresis, alteration of leukocyte count, altered hydromineral balance, and behavioral changes (see Handling and Transport, Section V). Although these responses can have positive as well as negative ramifications, generally they should be avoided. Tertiary responses are associated with long-term exposure to a stressor. Effects associated with tertiary stress include decreased growth, propensity to contract disease, and decreased reproductive function (Iwama et al. 1997; see Field Acclimation, Section V, and Acclimation to

Laboratory Conditions, Section VII).

Measuring and Avoiding Stress

While the nature of stress is insidious, it also tends to be polymorphic, changing with time and taking very different forms in different species at different stages in their lives. It is rarely appropriate or feasible to measure changes in blood hormones to assess stress; therefore, investigators are advised to design experiments that avoid stress unless the purposes of the research require measurements of stress indicators. Careful experimental design and detailed planning can ensure studies that are not confounded by unrecognized or unmeasured stress. Unless the aim of the research is to establish optimal conditions for holding particular species of fish in captivity, such as captive propagation of endangered species, it is generally advisable for researchers to select species for experiments whose optimal holding conditions are known and available. Species that have been held previously in the laboratory or that are commonly used in fish culture should be selected whenever such a choice is compatible with research objectives.

Environmental factors that are likely to cause stress and the stress responses typical for the species and life history stage should be understood well enough to design optimal holding conditions, transfer procedures and equipment, and experimental procedures so that stress will not result. Information on stress will more likely be available for those species that have demonstrated utility in laboratory or culture settings. Specific factors that should be considered and that frequently can be managed to minimize stress responses in fish include the following: (1) choice of species, (2) history of the animals under study, (3) water chemistry, (4) water flow, (5) water temperature, (6) light conditions and cycles, (7) bottom substrate, (8) noise and other physical stimuli, and (9) stocking density. Other variables, such as the presence or absence of tank covers, may be important in specific studies. We emphasize again the fact that these variables include items that are different from those typically considered for other animals, homeotherms in particular.

In addition to the aforementioned factors that are associated with long-term maintenance, several additional considerations apply when fish are handled or subjected to various experimental manipulations. (1) Handling should generally be minimized. Merely catching fish in nets can induce increases in stress hormones, such as cortisol, within 30 minutes. Although these effects can be cumulative, they need not be associated with damaging tertiary responses. Generally, fish should be given time to recover from handling before being used in experiments. The amount of time needed may vary with species and conditions; therefore, preliminary tests should be conducted to establish the appropriate recovery period. (2) The effects of stressors can be reduced through the use of anesthetics or adding salts to fish holding water. (Note that marine fishes usually will be an exception to this advice. Also, the specific salts and concentrations will vary depending upon species and environmental conditions.) However, anesthetics themselves can evoke physiological stress responses, so they should be employed cautiously and in

accordance with established guidelines. (3) The environmental conditions under which fish are held should not be changed rapidly, especially temperature conditions (e.g., an instantaneous change of 2°C in water temperature generally is not lethal but can cause detectable stress responses). Sudden temperature changes of greater magnitude, either upward or downward, are very stressful and should be avoided. The magnitude of change that fishes can tolerate will depend on the species, the life history stage in consideration, previous thermal history, and the initial conditions. Effects of previous thermal history have been detected for as long as a month post-treatment. Rapid, substantial changes in water quality also should be avoided (see Water Quality, Section VII). (4) If consistent with study objectives, fish should be given considerable time to recover from disturbances. At least 1 week of recovery is preferable, and 24 hours should be considered a minimum. Although physiological responses may return to pre-stress conditions more quickly, the fish may be abnormally sensitive to subsequent disturbances for longer periods of time. Resumption of normal feeding activity is usually a good measure of recovery. As experienced fish culturists and researchers have learned, the critically important axiom for successful maintenance of fish in captivity is: "know your fish." Inexperienced researchers are advised to work closely with experienced individuals until they gain sufficient experience to understand what is normal for their fish.

Readers may obtain additional information concerning stress and stress responses in fishes from several recent reviews (Iwama et al. 1997; Wendelaar Bonga 1997; Barton 2000).

Nociception and "Pain"

The question of whether or not animals other than humans experience pain as it is experienced and perceived by humans continues to be a hotly debated issue. This question is central to debates over the conduct of research on living animals and whether or not it is acceptable to conduct such research. While our UFR Committee is firm in its belief that research on live fishes is acceptable and essential, we recognize the difficult task facing IACUCs that must develop institutional guidelines that are functional and acceptable for their constituents. A complete, scientifically valid discussion of "pain" and the differences between "pain" and nociception in fishes is beyond the scope of these Guidelines. Nor can we provide a general policy that will be broadly applicable to all situations in all institutions; therefore, we advise individual researchers, the institutions in which they work, IACUCs, and regulatory bodies that it is essential that they develop policies concerning pain that are appropriate for fishes and consistent with the latest scientific understanding of pain and the neurobehavioral nature of fishes (Rose 2002). The following discussion provides information to assist the process of developing institutional policies that are appropriate for fishes and are scientifically valid.

Much of the confusion concerning pain in fishes, and the controversy that results from that confusion, is due to a failure to understand nociception and how it differs from pain. Pain is a psychological experience with both a perceptual aspect and an emotional aspect.

The term nociception is used to refer to the detection of injurious stimuli by the nervous system, injury which may or may not lead to the psychological experience of pain. Nociception is a universal characteristic of animal life. In contrast, pain is a psychological experience of the conscious mind. Consciousness is an essential prerequisite to the experience of pain. The nociceptive behaviors of fishes, the various reactions to injury or injurious or threatening stimuli, may appear to human observers to be similar to the behavior exhibited by humans experiencing pain. However, the term "pain" may be used appropriately only in reference to the unpleasant psychological experience that can result in the conscious mind from a nociceptive stimulus.

Is it possible to determine the presence or absence of pain when no verbal report of pain can be obtained? Modern brain imaging technology has made it possible to observe directly and to quantify neural events that relate systematically with the subjective experience of pain (Bromm 2001). Positron emission tomography, single photon emission computer tomography, functional magnetic resonance imaging, magnetoencephalography, and contemporary recording methods for electroencephalography have shown repeatedly that pain experience, consisting of both sensory and emotional components, is systematically associated with activity changes in specific cortical regions of the frontal and parietal lobes. Thus, essential neural processes that are responsible for pain experience in humans can be observed objectively, even in real time. Although some of these methods, in principle, could be applied to fishes, the effort would be fruitless due to fundamental differences between fish and human brain structure. The cortical regions that are essential for human pain experience, and whose activity can be observed with contemporary methods, are not present in the brains of fishes and there are no functionally comparable structures. This known dependency of the experience of pain on specific human cortical structures and the complete absence of these structures or functional equivalents in fishes is a principal point of evidence indicating that the psychological experience of pain is a neurological impossibility for fishes (Rose 2002).

Notwithstanding the information provided above, we recognize the issues of fishes' ability to experience "pain" will continue to generate controversy. A recent study (Sneddon et al. 2003) illustrates the ongoing controversy and the ease with which observations by competent scientists can be misinterpreted. The investigators observed the presence of C-fibers, the principle nociceptive receptor, in bony fishes; however, this fact has been known for more than 30 years. They were also able to demonstrate nociceptive behavior in rainbow trout *Oncorhynchus mykiss* injected with massive amounts of bee venom or acetic acid but not in fish injected with dilute seawater. They concluded that they had proved that fish experience pain; however, they failed to address in any way the requirement for demonstrating consciousness. Until conscious reaction to injurious stimuli can be demonstrated, we can only conclude that the behavior demonstrated by the fish in this study was nociceptive behavior.

We recommend that researchers pay careful attention to controlling and minimizing

physiological stress, because this will eliminate most nociceptive behavioral responses by fish that some observers may interpret as pain. We also recommend that the members of individual IACUCs study the recent review by Rose (2002) before developing specific policies, recommendations, and regulations concerning "pain" in their institutional guidelines.

V. Field Activities with Wild Fishes

Habitat and Population Considerations

Whether fishes are being collected for live study, preserved for study in a museum, or being processed to obtain data needed for fisheries management field studies, investigators should observe and pass on to students and employees a strict ethic of habitat conservation and respectful treatment of the animals (ASIH et al. 1987, 1988). Collecting should always be conducted in a way that minimizes habitat disturbance and "excessive" mortality. (The UFR Committee recognizes and accepts the fact that, at present, there is no field collection technique that will not cause any mortality in the population under study. Research goals will generally dictate appropriate sampling methods. Given a series of alternative methods and collecting gears, researchers should select the one that causes minimal disturbance and mortality in target and nontarget populations.) The collection of large series of animals from breeding aggregations should be avoided unless required to meet study objectives, as should the use of collecting techniques that damage habitat unnecessarily. Sampling equipment and strategies should be designed to minimize bycatch, the incidental capture of nontarget species. Regardless of the purpose of the experiment—whether to manipulate abundance or study behavior, reproductive potential, or survivability—disturbance should be kept to the minimum the investigator determines to be necessary to test the hypothesis accurately.

Collecting (General)

Research with fishes frequently involves capture of wild specimens from the field, whether for field activities, such as data recording, marking, and relocation, or laboratory study of live or preserved specimens. Except when collecting in the open ocean (waters not under the jurisdiction of any particular country), collection of fishes for all research purposes requires a scientific collector’s permit. Permits are issued by state, provincial, and federal natural resource agencies. Permit applications generally request information about the research to be conducted, sampling methods, and the areas and number of fishes to be sampled. For a listing of state permitting agencies in the United States, with addresses and fees, see Walsh and Meador (1998). Collection of fishes on federal lands often requires a separate special use permit obtainable from the agency that manages the land. The state and federal agencies that issue the permits require collectors to notify

them of the specific locations, dates, and proposed methods of sampling.

Systematists and taxonomists interested in conducting studies on preserved fishes should be aware of the wealth of material archived in natural history collections before removing additional specimens from the field. Additional, repeated collections, however, are often warranted to provide information on temporal changes in the health and well-being of the populations being studied. The holdings of many ichthyological collections are accessible on the Internet (e.g., University of Kansas Natural History Museum) or by contacting the curators at museums, such as the National Museum of Natural History, Smithsonian Institution, Washington, D.C.; American Museum of Natural History, New York; Field Museum, Chicago; California Academy of Sciences, San Francisco; Harvard Museum of Comparative Zoology, Cambridge, Massachusetts; Royal Ontario Museum, Toronto; or various other state and university natural history collections (for a listing of fish collections in the United States and Canada, see Leviton et al. 1985; Poss and Collette 1995; Walsh and Meador 1998).

Representative Samples

Generally, the questions being explored and the study design dictate the number of specimens required for an investigation. Sampling fishes for study generally involves the taking of a portion of the population or community present at the sampling location. The general principle that should be applied when sampling fishes is to take the smallest number of animals necessary to answer reliably the questions being posed. It should be emphasized, however, that where sampling is quantitative, the sample is based on a specific unit of effort applied or an area or volume of habitat sampled. For abundant fish species, such sampling may result in the capture of large numbers of specimens. Moreover, depending on the sampling method used and the amount of handing required to process the sample, such sampling may result in high mortality. This is especially true of sampling fish eggs and early life stages. However, high juvenile mortality and rapid recovery from population reductions are natural characteristics of the life histories of many fish species.

Collection of Imperiled Species

The term "imperiled species" applies not only to species listed officially as threatened or endangered by state or federal agencies but also to species that have been identified as candidates for such listings. It is important to know if an area to be sampled supports imperiled species and how to identify those species in the field (Warren and Burr 1994). We emphasize that collection of imperiled species should be avoided unless they are the subjects of the research being conducted. In many instances, imperiled species are protected by federal and state laws which prohibit collection except with special permits. If the goal of the research is to collect an imperiled species for live study, or if incidental capture of an imperiled species is a likely consequence of collecting other species, then collection techniques that are injurious or lethal (e.g., ichthyocides) should be avoided.

Appropriate state and federal permits must be obtained in advance. Lists of state-protected species may be obtained from offices that issue collection permits and from the web sites of NatureServe (originally known as the Association of Biodiversity Information) and the USFWS. The list of federally threatened and endangered fishes may be obtained from the Division of Endangered Species, MS 452, U.S. Fish and Wildlife Service, 4401 North Fairfax Drive, Arlington, Virginia 22203, USA; or NatureServe. Lists of protected marine or anadromous fishes are available from the Division of Endangered Species, National Marine Fisheries Service-F/PR3, 1315 East-West Highway, Silver Spring, Maryland 20910, USA). Lists of protected fishes in Canada (Canadian Wildlife Service or the Species at Risk Act Public Registry) and Mexico can be viewed electronically as well.

Conservation efforts for imperiled fish species frequently involve translocations, either among natural localities or from nature to propagation facilities then back to nature. The environmental laws governing translocations of imperiled fishes are complex and based on such matters as resource use, suitability and security of transplant sites, and appropriateness of transplanted individuals (i.e., sufficient numbers or freedom from disease; Minckley 1995). All translocation efforts must be conducted by the agency with authority and responsibility for the species and area in question and should not be attempted by individuals.

Museum Specimens and Other Preserved Specimens

The collection of fishes from natural populations for museum preservation is critical for (a) understanding basic biology and life history; (b) documenting and recording biodiversity; and (c) establishing reference collections essential for understanding evolutionary relationships and environmental impacts (ASIH et al. 1987, 1988). Studies of geographic variation and delineation of new species frequently require collection of relatively large series (sufficient for computing statistics on counts and measurements) from across the geographic ranges of species. Studies of molecular systematics typically involve very small numbers of specimens, or small amounts of tissue removed from study fish. However, it is just as important in these studies, as in general ecological surveys, to deposit voucher specimens in natural history museums for future reference (Wheeler 2003). Such samples should be frozen or preserved in 95 percent alcohol. The fact that the specimens are maintained in museums means that they are available for use in other types of research. Two important principles that should be followed in collecting fish for museum preservation are: (1) the numbers of specimens collected should be the minimum necessary to accomplish study goals; and (2) each animal collected should serve as many types of study as possible.

Specimens collected for museum deposition should be preserved in a manner that maximizes their utility for study and minimizes the need for additional collecting. Formalin fixation is the standard practice used to ensure long-term preservation quality of fish specimens. The preferred method for archival storage is direct immersion in a 10%

formalin (formaldehyde) solution, followed by transfer to alcohol for long-term preservation. Chemicals are sometimes added to formalin to buffer the solution or to preserve color (e.g., Ionol) (Fink et al. 1979). Although formalin is the fixative of choice for vertebrate tissues, other fixatives are sometimes used for specialized study purposes such as histology (Bouin's or Gilson's fluid) and electron microscopy (glutaraldehyde). Fixation by these methods typically involves small pieces of tissue dissected from specimens that may be sacrificed by means other than immersion in formalin. However, if the carcasses will be archived as voucher specimens in long-term storage, they should be fixed in formalin and transferred to alcohol.

Euthanizing fish prior to immersion in formalin should be considered, provided that the anesthetic does not cause effects detrimental to the objectives of the research. A variety of chemicals may be used to anesthetize or euthanize fish (see Restraint of Fishes: Anesthetics and Related Chemicals, Section VII). When study interests demand that specimens be fixed without prior treatment with anesthetics, the specimens can be numbed in ice water or, for fishes less than 10 centimeters in length, immersed directly in liquid nitrogen.

Live Capture Techniques and Equipment

The choice of a sampling method should be dictated by worker safety, research objectives, seasonal considerations, and the habitat type to be sampled. Capture techniques should prevent or minimize injury to study animals (McMichael et al. 1998; Henry and Grizzle 2003; Henry et al. 2003). Live wells or tanks should be provided if fish are to be kept for more than the time needed to take essential measurements. Care should be taken to avoid accidental capture of nontarget species and to insure release with minimal injury (ASIH et al. 1987, 1988). Species that may be dangerous to workers due to size or species-characteristic behavior or capabilities require additional precautions (see Dangerous Species and Specimens, Section V, Dangerous Species and Specimens (2), Section VII).

Several studies have shown electrofishing to be the most effective technique for obtaining fish assemblage data in freshwater habitats (Yoder and Smith 1999). Pulsed direct current electrofishing can be performed by wading methods or boat-mounted methods. Appropriate electrofishing protocols should consider the sampling purpose and physical constraints of the environment, salinity, water depth, and presence of snags. Alternative sampling methods include seining, gill nets, hook and line, lift nets, slat traps, hoop nets, angling, and snorkeling. The sampling method chosen should provide a relatively accurate estimate of abundance with operational efficiency, accuracy, and minimal injury, as well as a reasonable cost (Nielsen 1998). If certain species or sizes of fish are needed, it is desirable to select a sampling method that will not collect nontarget species from the entire aquatic community. A decision tree can be followed to decide which method is the most appropriate. Murphy and Willis (1996) provide additional information concerning

the efficiency and specificity of various collecting gears.

Field Restraint of Fishes: Anesthetics

Prolonged restraint under conditions that cause physiological stress should be avoided. In some cases, utilization of general anesthesia for restraint may be advisable. The only substance approved by the FDA for field anesthesia of fishes is tricaine methane sulfonate (MS-222). However, use of this agent in the field is limited due to an FDA requirement that food fish, including feral fishes that may be caught and eaten, must go through a 21-day (minimum) withdrawal period prior to release (Anderson et al. 1997). Addition of an appropriate buffering compound, such as sodium carbonate, to MS-222 is recommended to increase post-treatment survival (Smit et al. 1979). One or two fish should be tested to ensure that the species will return rapidly to normal physiological and behavioral status. The animals must be kept under observation until appropriate recovery occurs. Disposal of used anesthetic must be done in accordance with local, state, and federal regulations (see also Physical Facilities for Temporary Holding and Maintenance, Section V, and Restraint of Fishes: Anesthetics and Related Chemicals, Section VII).

Dangerous Species and Specimens

Most frequently, dangerous species will be encountered only under field conditions; however, the recommendations that follow are equally applicable to laboratory situations. Dangerous species should be handled in a manner that is safe for both the investigator and the animal being handled. Researchers should be cognizant of the safety regulations of their institution regarding the use of dangerous or venomous animals. Those regulations may include SOPs that limit access to dangerous animals to only authorized individuals, specify use of protective clothing or handling devices, and dictate treatment of individuals injured by the animals, including first aid and procedures for obtaining follow-up medical care. Special handling methods will depend upon the species being handled, the nature of the danger to the investigator, and the nature of the research effort. Adherence to the following general guidelines is recommended:

(a) Procedures should minimize the amount of handling time required and reduce or eliminate contact between handler and animal.

(b) The researcher should never work alone. A second person, who is knowledgeable in capture, handling techniques, and emergency measures, should be present at all times.

(c) Prior consultation with colleagues who are experienced with the species, as well as review of any relevant literature, is of particular importance. Much of the information on handling dangerous species has not been published but has simply been shared among investigators working with these species.

Handling and Transport

Fish will exhibit some degree of stress response when handled and transported. Methods of handling fishes vary with the species, the environment in which they are found, and the tradition and resources of a particular region or country (Avault 1996). Stress responses can be reduced, however, by eliminating rough handling, rapid temperature changes, sudden changes in water quality, abrasion, and excessively tight confinement. Inappropriate handling and transport procedures can contribute to changes in blood profiles (Ellsaesser and Clem 1986) and substantial mortalities (Weirich 1997; Carmichael et al. 2001). Handling and transport procedures must be designed to minimize the effects of stress and thereby reduce immediate and delayed losses.

Some physiological changes that occur in response to handling and transport stressors are measurable and can be monitored. These changes include increased cardiac output, increased gill vascularity, and release of catecholamines and corticosteroid hormones (Carmichael et al. 1984a; Weirich 1997). Handling of fish in the field or in the laboratory is frequently characterized by increased susceptibility to disease thought to be mediated by immunologic suppression (Wedemeyer 1970). Lymphopenia, neutrophilia, and lymphocyte nonresponsiveness have been noted as results of handling and transport stress (Ellsaesser and Clem 1986). Clinical hematological values are available for some species (Stoskopf 1992a). Depending on the severity of the stressors and exposure time, mortality usually results from osmoregulatory dysfunction and immunosuppression. To mitigate stress associated with handling and transport, one can reduce the number and severity of the stressors, minimize the duration of stressors, and minimize increases in metabolic rate. Harvesting techniques and pre-shipment treatment are important to the successful shipping of live fish (Dupree and Huner 1984). Pre-conditioning treatments can involve the addition of anesthetics to reduce metabolic rate or salt or calcium to the transport water to prevent or reduce osmoregulatory dysfunction and resulting ionic imbalances (Carmichael et al. 1984b). Feed can be withheld for 1 or 2 days prior to transport (Weirich 1997). Generally, transports are less damaging to animals if done in cool weather. Proper equipment for transport should be used.

Transport tanks should be well constructed and should be disinfected before use (Avault 1996). The weight of fish that can be transported safely in a live-hauling vehicle depends on efficiency of the aeration system, duration of the haul, water temperature, fish size, and fish species (Avault 1996). Maintaining acceptable levels of dissolved oxygen, carbon dioxide, temperature, ammonia, and pH during transport is essential. Fish can be transferred between capture and transport units, or between transport units and holding units, by wet or dry transfer methods. Wet transfer involves transport of fish in a container of water and minimizes direct contact with nets. Wet transfer usually results in less stress than does dry transfer, where the net is used alone. Ideally, fish should be allowed to recover in the same or similar medium used for transport (Carmichael et al. 1984b; Weirich 1997). The length of time for recovery may be variable depending upon conditions, the amount of handling, and research objectives, but 72 hours typically is

considered a minimum following extensive handling (see additional information below under Field Acclimation, this Section).

Physical Facilities for Temporary Holding and Maintenance

Because the biological needs of each species and the nature of individual projects vary, only the most general recommendations on temporary holding and maintenance can be made. When dealing with unfamiliar species, testing and comparing several methods of housing to find the most appropriate for the needs of the animal and the purposes of the study may be necessary. Ease of maintenance by animal keepers, though important, should not be the prime determinants of housing conditions; however, such ease generally ensures greater compliance with established maintenance protocols (ASIH et al. 1987, 1988).

Normal field maintenance facilities should incorporate those aspects of the natural habitat deemed important to the survival and well-being of the animal. Adequacy of the maintenance facility can be monitored by observing changes in growth and weight, survival rates, activity levels, general behavior, and appearance (Snieszko 1974). Nutritionally balanced diets should be provided or natural foods should be duplicated as closely as possible. Natural light and temperature conditions should be followed unless alteration of these are factors under investigation (ASIH et al. 1987, 1988). Frequency of tank cleaning should represent a compromise between the level of cleanliness necessary to prevent disease and the amount of stress imposed by frequent handling (ASIH et al. 1987, 1988).

For culture, bait, or sport fish species, fish are usually held in vats or tanks before they are shipped. This holding enables the producer to grade fish according to size and to apply the appropriate parasite removal treatments. Holding also acclimates them for handling and transport (Huner et al. 1984). When harvesting from a pond, live cars or fish holding bags are used in the industry for channel catfish *Ictalurus punctatus* (Huner et al. 1984) and can be coupled with a harvesting seine to serve as temporary holding containers and graders. These methods generally are applicable to all pond-reared species. In pond holding situations, it is beneficial to move the fish to deeper water and to use recirculating pumps or aerators, if needed.

As with other containment systems, the holding tank needs to take into account the stocking density or the relationship of fish biomass to available water volume. Water inflow and turnover time must be taken into account because sufficient water exchanges are needed for good water quality. Oxygen available in the incoming water needs to exceed the metabolic oxygen consumption of fish in the tank (Casebolt et al. 1998). Sufficient aeration can be supplied by compressed air, bottled oxygen, or agitation. Anesthetics can also be used to reduce the physical activities of fish, if consistent with research objectives. Excess noise and vibrations that are not present normally should be avoided because such factors can produce acute or chronic stress response in fish

(Stoskopf 1992b).

Field Acclimation

Because myriad physiological processes are altered upon handling and transferring fish, an effort should be made to acclimate or condition fish to their new environment. If the physical and chemical qualities of the water supply for the temporary holding facility are different than those of the water from which the fish were taken, care should be taken to provide water as similar as possible. Plastic bags, with an atmosphere of oxygen over the water, may be used to allow the captured fish to acclimate to the new temperature conditions. Gradually replacing the water in transport units with water from the source for the holding unit is a commonly used practice to provide time for acclimation to new temperature and water quality conditions. However, this practice should be used only when the differences between the water from which the fish were taken and the water in the holding or transport unit do not exceed tolerance limits for the species. Useful notes on how to transport and acclimate live freshwater fishes may be found at the Maryland Sea Grant Research web site.

Collection of Blood and Other Tissues

Results obtained from careful collection and examination of blood and body fluids are often critically important to research on fishes (Blaxhall 1972; Fange 1992). Sterile conditions for these procedures are impossible to provide under field conditions; however, care must be exercised to prevent injuries and stresses that are not essential to the collection of the tissues or fluids. Samples of blood and body fluids can be obtained from fish without compromising their survival, even from small specimens under 100 grams (Stoskopf 1992a). Plastic syringes containing a small amount of anticoagulant, such as lithium-ammonium heparin or sodium citrate, can be used to avoid clotting. Study objectives will determine the proper selection of type, volume, and concentration of anticoagulant. Three main techniques have been devised for collecting blood from fish: cardiac puncture, venous puncture, and caudal bleeding (Blaxhall 1972; Stoskopf 1992a). The tail is the preferred site for blood sampling. The vessels running beneath the vertebrae of the fish can be sampled by using a lateral or ventral approach. Cardiac punctures from the ventral side are sometimes used in fusiform fishes or through the operculum in laterally compressed species. For repeated sampling, cannulae have been implanted in the dorsal aorta through the buccal cavity. Blood from the caudal vessels may be collected directly into collection tubes by cutting off the tails of anesthetized fish that will be euthanized following the procedure. However, extraneous fluids and proteins may be collected with this procedure. Caution must be exercised to ensure that the method of anesthesia will not interfere with subsequent analyses. Additional information on sampling methods for the collection of blood from fish and the various anesthetic agents used has been described by Klontz and Smith (1968), Smith et al. (1999), and Marino et al. (2001).

Additional tissues that are useful for collection include otoliths, gills, kidney, thyroid, spleen, testes, ovaries, liver, heart, brain, and muscle. Collection of internal tissues normally requires sacrifice of the subject animals and must be preceded by appropriate anesthesia or euthanasia. These tissues can also be used in biopsy or necropsy and may be examined prior to fixation and histological processing. Depending on the purpose for the sample, tissues may be used fresh, frozen, or placed in a fixation or preserving medium such as buffered formalin, ethanol, or methanol (Luna 1968, 1992; Humason 1979). The purposes of some studies may be served by collections of scales, spines, or small pieces of fin, which can be accomplished with minimal effects on the fish from which they are taken.

When transporting live tissues, the medium must have appropriate ionic and osmotic concentrations and contain a sugar as an energy source. Experienced researchers have found Hank's Solution, Earle's Balanced Salt Solution, or Holtfreter's Solution to be effective transport media (Holtfreter 1931). Noncytotoxic antibiotics or antimycotic agents may be included to prevent the growth of bacterial and fungal organisms.

It is becoming increasingly common to remove small pieces of fin tissue to obtain DNA for sequencing and other types of molecular studies. Pieces of fin preserved in 70% or higher concentrations of ethanol can yield adequate amounts of DNA, and the fin clips are not harmful to the fish. This technique is especially important when working with imperiled species and small populations.

VI. Marking and Tagging

General Principles

Tags and marks have been used to obtain information on the biology of tagged organisms and to develop rational management strategies. The identification of fish over time is required for studies focusing on ecology, fish behavior, age, mortality rates, abundance, population dynamics, migrations, stock identification, and stocking success (Wydoski and Emery 1983; Buckley and Blankenship 1990). Major advances in tagging methodologies occur when investigators apply their understanding of the target animal's biology to the design or modification of a tag (McFarlane et al. 1990). Many tags and marks developed initially for freshwater, marine, or anadromous fishes have been applied subsequently to other species regardless of habitat. Researchers can use both intrinsic and extrinsic identification systems, where the nature of the study dictates the type of tag or mark employed. Integrated use of more than one tagging or marking technique helps to ensure fish identification.

Prior to marking fish, investigators must consider the amount of tissue affected, and whether or not the effects of handling will be momentary or prolonged. Investigators

should also determine if the animal will be at greater than normal risk to predation, if its desirability as a mate will be reduced, and if a risk of infection is increased substantially (ASIH et al. 1987, 1988). Marking techniques for fishes have been extensively reviewed and are constantly evolving; therefore, investigators should review recent literature (Wydoski and Emery 1983; Emery and Wydoski 1987; McFarlane et al. 1990; Parker et al.1990; Nielsen 1992).

External Tags, Marks, Biotelemetry

External tags and marks have evolved over a long period of time (McFarlane et al. 1990). Basic considerations for selecting an external mark or tag are the objectives of the study; effect on survival, behavior, and growth; permanency and recognition of the mark; number and size of the organisms to be marked; stress of capture, handling, and marking; cost; recovery of tagged fish; and coordination required among agencies, states, or countries (Wydoski and Emery 1983). Extrinsic methods most commonly used for fisheries research involve natural tags or artificial tags, and each type has different capabilities and limitations.

Natural biological marks include meristic, pigmentation, morphometric, and scale characteristics, but their use is limited because they are subjected to environmental and genetic influences. The shape, size, and circulus patterns of scales are the most frequently used natural marks. Using biological marks generally requires much knowledge about the life history of the organism.

Multiple artificial tagging methods are available. Alteration of fins or other body parts has been in practice for over 100 years and is accomplished by clipping or hole-punching fins or other body parts. Fins selected for clipping or removal can depend upon the species selected; for example, clipping the anal fin of poeciliid males would be inappropriate, but removal of the adipose fin of a salmonid would have negligible impact (ASIH et al. 1987, 1988). Hot branding or cold branding, the processes of marking an organism by placing either a hot or cold instrument with written characters against the body for a few seconds, may be effective marking techniques in specific situations and do not cause substantial injury to underlying tissues in fishes.

The development of physical tags has offered much versatility to answer biological questions. Tags are conspicuous by their color, shape, size, or attachment location and are made from a variety of materials. The dye and pigment category includes dyes, stains, inks, paints, liquid latex or plastics, metallic compounds, tetracycline antibiotics, and radioactive isotopes that are administered by immersion, injection, tattooing, or feeding. Technological advances in design of tags and tagging systems will continue to evolve. Interpretation of tagging data can be affected by tag loss and the failure to report recovered tags. Data logging tags may present unique problems because of large size and potential effects on the ability of fish to swim and feed normally.

Underwater biotelemetry involves attachment of a device that relays biological information via ultrasonic or radio signals from a fish to a remote receiving system (Winter 1983, 1996). Radio transmission is practical only in freshwater at relatively shallow depths (AISH et al. 1987, 1988). The selection of a tag or transmitter and the method and site of attachment or implantation should be appropriate for the species and size of fish under consideration to meet the objectives of the project. Personnel should be appropriately trained in specific tag implantation.

The effects of marking on the animals depend on the physical condition of the fish at the time of release. Occurrence of injury is species and size specific, where particular species and smaller fish are more susceptible than others. Wounds that are caused by marking normally heal satisfactorily without the use of antibiotics; however, unnecessary or inappropriate marks and tags may provide inaccurate data and, therefore, should be avoided. All anesthetics or antibiotics administered must be used in a manner consistent with government regulations.

Internal Tags and Marks

Implanted wire tags, passive integrated transponder (PIT) tags, otolith marks, and natural parasites are internal marking systems used to identify fish (Prentice et al. 1990). The coded wire tag identification system has been tested for management and research applications with multiple genera of fishes (Buckley and Blankenship 1990) and does not cause adverse tissue reactions. The coded wire tag is normally injected into cartilage, connective tissue, or muscle and is later detected electronically with a hand-held device. The use of transparent tissues as injection sites can decrease the necessity for external indicators. Shallow implantation of tags facilitates benign surgical recovery of the tags.

The PIT tags consist of small computer chips that are injected into specimens for permanent identification. These tags are assumed to be reliable for the duration of the lifespan of the fish (Freeland 1995). These tags can be read easily through soft and hard tissue, seawater, freshwater, glass, plastic, metal, and when tags are moving at some velocity (Prentice et al. 1990). The reading device may be powered by alternating current or battery for convenient use in the field as well as the laboratory. Information may be downloaded directly to a computer.

Manipulations of environmental temperature, feeding rates, photoperiod, or external chemical baths can induce specific marks in fish otoliths. Otolith microstructural features are permanent and can be viewed and analyzed in fish of any age. Tetracycline and other fluorescent compounds are well-known markers for calcified structures in fish (Brothers 1985). (Such treatments are regulated by the FDA as a drug treatment.) A strength of this system is the ease of application to otoliths at any time during the growth period of the fish. Fish that are propagated under controlled conditions are readily available for such manipulations. Fisheries that require stock definitions and assessment of success of

stocking early life stages benefit from the otolith marking system.

Several taxonomic groups of fish parasites have been used as biological tags, and this method is best suited to the separation of relatively self-contained stocks of fish (MacKenzie 1983). Recovery of internal parasites used as biological tags is enhanced if parasites are associated with a specific anatomical site of the fish. The decision to use a parasite as a natural mark on fish is determined by calculating the ratio of incidence of that parasite on one fish population to its incidence on another (Wydoski and Emery 1983).

Genetic Markers

The development of techniques employing markers based on chromosome and nuclear DNA polymorphisms has been very rapid. The potential uses for such markers in selective breeding programs, evaluating the contribution and effects of stocked species, or delineating specific habitat requirements have emerged quickly. The fact that adequate samples of the tissues needed for analyses of such markers can be obtained nonlethally (e.g., fin clipping) and with minimal handling provides additional incentives for their use in a wide array of studies.

The use of genetic markers is not new, but prior to the development of recent DNA techniques, allozymes were used as the genetic markers to differentiate fish populations. The use of these markers required the examination of dividing cells and, in some cases, killing fish to obtain appropriate samples. The use of DNA markers for fish stock identification was initially limited to differences in mitochondrial DNA (Phillips and Ihssen 1990), but newer technologies, such as restriction-fragment-length polymorphisms and random amplified polymorphic DNA, allow for screening for genetic variation to obtain different molecular fingerprint patterns. Small laboratory fishes such as Japanese medaka *Oryzias latipes* and zebrafish *Brachydanio rerio* (also known as zebra danio *Danio rerio*) have been used extensively as models for studies in vertebrate developmental genetics and for transgenic investigations (Ozato and Wakamatsu 1994). The genetic structure of specific fish assemblages can contribute to biomonitoring programs (Gyllensten and Ryman 1985).

In the face of questions concerning stocking programs and native species, the use of molecular techniques can provide additional information to address these issues. Genetic markers will be valuable for managing performance traits such as long-term reproductive success and assessing habitat restoration. Fisheries scientists dealing with such questions will need to update their knowledge of the most current, scientifically accepted genetic identification systems and their potential applications (Lincoln 1994; Poompuang and Hallerman 1997).

Isotopes

The use of stable isotopes, such as 13C, 15N, or 34S, as marks to identify places of origin, nutrient pathways, feed efficiencies, and an array of physiological or ecological processes is becoming relatively common. Stable isotopes occur naturally, behave identically to the "typical" isotope, and can be identified with a high degree of accuracy and reliability. Variation in the ratios of the stable isotope to the more common form can be used to identify sources of materials and to trace them within individual animals, populations, or ecosystems. In contrast to radioisotopes, the uses of which are regulated very tightly, the use of stable isotopes does not require special facilities and permits. (Information on potential uses of radioisotopes have not been included in these Guidelines because the detailed, specific information needed to comply with regulations would exceed the space limitations for this document.) Depending upon the objectives of the research, nonlethal sampling is possible, by using scales or fin clips for stable isotope analyses. Different tissues have different elemental turnover rates; therefore, each researcher must determine which tissues may provide materials needed to satisfy the requirements of their studies. Representative information on the use of stable isotopes have been provided by Peterson and Fry (1987) and Wada et al (1991).

VII. Laboratory Activities with Fishes

General Principles

Working with live fish under laboratory conditions requires attention to many details concerning the requirements and limits of tolerance for each species. Acceptable physical facilities and an adequate supply of appropriate quality water must be provided even if the fish are to be held only for short periods of time. Although fish may tolerate marginal facilities and conditions for a few hours or even several days, holding them under less than optimal conditions will affect the results of the research. Standards for humane treatment of animals must also be maintained, regardless of the length of time that the fish are held.

Confinement, Isolation, and Quarantine

Prior to bringing fish into a laboratory, facilities and plans should be in place to ensure that the fish cannot escape from the facility, especially species not native to the watershed, and that the introduced fishes can be isolated physically from fishes already present. Each holding unit should have its own set of nets and other equipment. Facilities and equipment used for previous studies should be disinfected prior to use in new studies, typically with a chlorinated disinfectant. If the introduced fishes may carry disease agents, especially pathogens or parasites that are not endemic to the area, quarantine-level facilities should be used. The level of quarantine required will vary with the seriousness of the known or suspected disease agent.

Experimentation with nonindigenous fishes, transgenic fishes, or other genetically modified fishes is a special situation that requires additional precautions to preclude the escape of such animals. The specific barriers may be similar to those used to prevent the escape of disease agents but must be developed to fit the physical characteristics of the laboratory or experimental facility. The USDA has developed specifications for its own facilities and published voluntary guidelines (USDA 1995a, 1995b) intended to ensure appropriate consideration of the potential genetic and ecological effects of such research activities. These publications assist in determining appropriate procedures and safeguards so that research can be conducted without causing potentially adverse effects on the environment. Suggestions are provided for developing facility inspection guidelines and risk management procedures, appropriate siting, construction of containment structures, and nonstructural containment strategies. Institutional guidelines for work with transgenic or other genetically modified animals must be variable to adapt to site-specific and study-specific activities but should be sufficient to ensure that accidental release cannot occur during floods, other natural disasters, or equipment failures. Ultimately, individual scientists are responsible for ensuring the containment of animals that may cause adverse effects on free-ranging populations of fishes or the environment.

Effluents from units used to hold newly introduced fishes should, at a minimum, pass through screens with openings sufficiently small to retain any escaped fish, followed by mechanical grinding devices and, in turn, chemical or other treatment sufficient to kill all pathogens and parasites that can be expected to be present. Facilities conducting research on controlled disease agents (see OIE lists) must have isolated, self-sufficient units for the conduct of the research and must restrict access of unauthorized individuals to these units. In addition, physical barriers must be in place with sufficient capacity to prevent outflow of any water in the event that all holding units are emptied (USDA 1995a, 1995b).

Acclimation to Laboratory Conditions

Fish should be given time to acclimate to new environments, feeds, and routine activities before being used in studies. Slow acclimation to change often is critical (Casebolt et al. 1998). It is not uncommon for fish to exhibit acute health problems 48 to 72 hours following transfer. The time used for acclimation within and between experiments should be standard and specific for a species. Preliminary studies may be needed to establish the most appropriate time to be used during individual studies. A commonly used acclimation period is 2–4 weeks. However, investigators should note that laboratory holding conditions may cause physiological changes in wild animals brought into the laboratory, such as immunosuppression, or loss of tidal or diel rhythmicity, even though no visual signs of stress are present. For example, Miller and Tripp (1982) described a differential inhibitory effect on lymphocyte subpopulations in such animals (see Stress, Section IV).

Physical Facilities (Permanent)

Laboratory culture systems are based upon a variety of designs, ranging from a few aquaria to large systems with a full complement of aquaria, raceways, and ponds. The numerous fish species have a variety of requirements; therefore, the laboratory should be designed to be flexible and to accommodate all species of potential interest. Generally, water supplies are flow-through systems of freshly input water; however, well-designed and operated recirculation systems can maintain water of adequate or even superior quality.

Culture systems will vary according to the physical size of the lab, availability of water, the species chosen for research, the number and density of test animals, and cost considerations. Fishes can be raised successfully in many types of systems, but there are optimum conditions for each species. In the design of a laboratory culture system, minimizing stress should be the paramount factor to consider to assure quality research animals. Adequate water flow, with consideration to both volume and flow patterns to provide adequate dissolved oxygen and to flush metabolic waste products, is one of the most important considerations (Piper et al. 1982). Consideration should also be given to eliminating, or at least reducing, the potential spread of disease agents within a system. Not only should such items as nets and other laboratory equipment be suspect as vehicles for pathogen transmission, but airborne movements of aerosols containing pathogens are also important means by which fish pathogens may spread (Wooster and Bowser 1996; Bishop et al. 2003).

Facilities that are poorly designed and constructed can hinder research activities because they cannot maintain the required quality, number, sizes, or species of fish. Water of excellent quality and quantity may be rendered useless for fish if pipes and valves release heavy metals or other contaminants into the water (Brauhn and Schoettger 1975; also see Water Quality, this Section). Researchers must be alert to the possibilities for long-term chronic effects of toxic materials on the physiology and behavior of captive fishes. There are many construction materials available that minimize contact with potentially toxic substances. Good components for construction of holding systems (tanks, valves, delivery lines, and drains) for culture facilities include glass, type 316 stainless steel, nylon , fluorocarbon plastics, concrete (ASTM 1998), polyethylene sheeting, rigid PVC, Teflon, and fiberglass (U.S. Army Corps of Engineers 1991). Brass, copper, lead, zinc, and rubber should be avoided (ASTM 1998). The chemical content and solubility of cements or other bonding products should be reviewed to determine the possible presence of toxic substances. Regular monitoring of water quality is essential. Systems designed for saltwater fishes will require additional attention to factors related to salinity and potential effects of corrosion, but the same general design considerations discussed above are applicable.

Density of Animals

The density with which fish can be held in an experimental unit depends on a series of environmental factors and also the behavioral characteristics of the species. The most

immediate concern is maintaining a supply of dissolved oxygen that is appropriate for the species, the temperature of the water, and the elevation (Piper et al. 1982). Accumulation of waste products, especially ammonia, is generally the next factor limiting density (Piper et al. 1982). Interrelationships of density, physiological stress, susceptibility to disease agents, and transmission of disease agents are additional factors that must be considered when density levels are established.

Oxygen demand and excretion of ammonia are directly related to the amount of feed supplied to the fish. The amount of feed is in turn determined by the number and the size of the fish in the unit. In general, flow-through systems can sustain a greater density of fish than can static units due to continual replenishment of dissolved oxygen and removal of ammonia. However, the bead filter technologies used in new recirculation systems have improved the densities that can be maintained in these systems (Malone and Beecher 2000; see Water Recirculation Units, this Section). Static units must be equipped with aeration and filtration equipment if the density of fish is to be more than the minimal levels that can be sustained through direct atmospheric exchange. In general, it is desirable to maintain dissolved oxygen concentrations near saturation and, for most species, never below 5 milligrams per liter. Ammonia concentrations should be near zero, especially at higher pH levels.

Fish vary from species to species, and even within species, as to the degree of crowding that they will tolerate before their behavioral patterns are disrupted. No specific guidelines can be provided, but the potential effects of crowding should be included in each research design (Piper et al. 1982). For the most part, practical density is determined by the water treatment system and feed delivery system and reaches the maximum at the density determined by social interactions. This "social point" can be very high in schooling species, assuming dissolved oxygen levels, other water quality factors, and feeding problems have been addressed. Investigators and IACUCs are cautioned to recognize that appropriate densities for various species and specific studies are variable. No standard, preferred density applies to all species.

Feeds and Feeding

Although most species of adult fish can survive several weeks without food, especially at lower temperatures, they must be provided with food that is acceptable to them and that will provide basic nutritional requirements, within a few days if they are to remain in satisfactory condition as research subjects. Migratory fishes may be exceptions to this general rule. A review of the life history of each species will provide the information to determine feeding requirements. If the nutritional requirements for the species and life stage are not known, a balanced mix of items found in the diet of free-ranging individuals of the species should provide adequate nutrition for longer periods of time. It cannot be assumed that supplying live prey, especially of a single species, will meet the complete nutritional requirements of the captive fishes. Some piscivorous species can be trained with relative ease to accept formulated feeds, thereby eliminating the problems inherent

in providing live food.

Formulated feeds can be expected to provide the nutritional requirements of the species for which those feeds were designed, especially if manufactured to the specifications of a specific list of ingredients (an open formula). Although captive fish frequently will consume feeds designed for other species, their requirements may not be met if such feeds are used for extended periods of time. Commercial formulated feeds usually are not based on a specific list of ingredients (closed formulas) but, rather, are designed to meet the broad nutrient requirements for protein, carbohydrates, and fats. Specific ingredients selected to accomplish the proximate analysis listed on the feed container can vary considerably from batch to batch, even though the relative amounts of major ingredients (protein, carbohydrate, fat) remain constant. As a result, the capability of these feeds to meet specific nutrient requirements is variable. Investigators must consider the possible effects of variability in ingredients on the physiology of their experimental subjects when the studies are designed (Barrows and Hardy 2001).

The amount of feed to be provided will vary with the nutrient and energy content of the food as well as the age and size of the fish. Feeding to satiation is the normal practice unless the research design requires lesser amounts. Fish that are maintained on live feeds typically should be fed to satiation. The weight of formulated feed to be fed, per manufacturers' instructions, is generally lower than that required for live feeds. Typically formulated feeds are fed at levels ranging from 3% to 8% of the weight of the fish, depending on water temperature and the species, size, and age of the fish.

Optimal feeding times depend on species-specific behavior but generally can be modified to accommodate the schedules of the fish caretakers. If the species of fish typically feeds at night or at dusk and dawn, it is desirable to provide feed at the times when they would feed naturally. Most formulated feeds can be dispensed by a variety of mechanical feeders or demand feeders triggered by the fish. Feeding by machine prevents habituation by the fish on "the hand that feeds them" and allows flexibility in feeding schedules. Excess uneaten feed should be removed from the tank within a short time following feeding. Water quality will be diminished by accumulated feed and water-soluble nutrients will be leached from the water-soaked pellets.

Water Quality

Providing water of appropriate physical and chemical quality is probably the most important single factor for the care and maintenance of captive fishes. Inasmuch as each of the 25,000+ species of fishes has its own optimum conditions and limits of tolerance, each investigator is responsible for determining the preferred conditions for the species under study. Transferring fish into water having a temperature outside their limits of tolerance, or even in excess of their capacity for change, can lead to death, either immediately or delayed, usually within 72 hours. Sudden changes in water temperatures as small as 5°C can cause serious stress responses in fishes that are otherwise healthy.

Most experienced researchers do not routinely expose fishes to temperature changes greater than 2°C. Limits of tolerance and ability to tolerate changes in temperature are influenced by the previous thermal histories of individual fish as well as species characteristics (Carmichael et al. 1984a).

The presence of toxic substances in water or the absence of sufficient dissolved oxygen can cause immediate death to fish placed in such water. Chronic water quality problems, such as elevated nitrite levels, may not cause obvious reactions but can seriously affect the physiology of the fish and research results. Prior to introducing fish, water supplies used to hold fish should be analyzed in detail for parameters, such as hardness, alkalinity (buffering capacity), major cations (Na, K, Ca, Mg), major anions (CO3, Cl, HCO3, SO4), heavy metals, and pesticides, prior to the introduction of fish into holding units receiving the water. In addition, routine monitoring of temperature, dissolved oxygen, ammonia, alkalinity, nitrite, and pH should be conducted. In the case of "soft" waters that are poorly buffered, substantial changes in pH may cause adverse effects on animals held in such water. Unionized ammonia is quite toxic to fishes and can cause stress or even mortality, especially at higher pH levels. Careful monitoring and addition of buffering agents may be warranted in such situations. The effects of temperature and elevation on water quality parameters must be known and managed to maintain conditions within acceptable limits (Boyd 1985; Avault 1996; Colt and Tomasso 2001).

If the water supply is city water or any supply that has been chlorinated, it should be monitored regularly, especially for free chlorine, which can produce immediate toxic reactions. Dechlorinating equipment, such as activated charcoal filters, and chemicals are available that can reduce free chlorine to undetectable concentrations; however, very low concentrations of chlorine by-products or metabolites may remain in the water. Normally these chemicals do not cause any short-term adverse effects; but the potential effects of such by-products and metabolites should be considered, and their concentrations monitored, if they could affect results.

Water Recirculation Units

The emergence of water recirculating systems in aquaculture over the past three decades has provided several benefits to fish culturists and researchers. The substantial water supply requirements and specific climatic conditions required by traditional fish culture systems are eliminated; fish can be produced and maintained year-round, and environmental impacts of organic effluent discharges are reduced (see Effluents, this Section). Recirculating technology has been employed for continuous loading with very high fish densities (Van Gorder 1991; Malone and Beecher 2000) and other purposes, such as hatcheries for prawn *Macrobrachium* sp. and broodstock maturation (Millamena et al. 1991).

The efficiency of a recirculating system depends on the components used in its design. Typically, each system will include units with capabilities for biofiltration, clarification

of solids, aeration, pH control, reduction of biological oxygen demand, water circulation, and maintenance of appropriate alkalinity, ammonia nitrogen, and nitrite nitrogen levels. The biological filter, or biofilter, is the central component in recirculating aquaculture systems. Additional components may include pumps, tanks, clarifiers, aeration and oxygenation capabilities, UV light or ozonation, and sumps.

Several types of biofilters are available. Those with the highest nitrification efficiencies function best to control the ammonia and nitrite levels. Nitrification is the process of ammonia removal and consists of successive oxidation of ammonia to nitrite and finally to nitrate, accomplished by bacteria in such genera as *Nitrosomonas*, *Nitrospira*, and *Nitrobacter*. In order to establish an active nitrifying bacterial population, it is necessary to precondition the biological filter for a period of several weeks prior to stocking with high fish densities. It must be noted that the maximum nitrification capacity is lower in saltwater systems than in freshwater systems; however, adaptation of freshwater biofilters to higher salinities can provide a tool for shorter start-up of a seawater system (Nijhof and Bovendeur 1990).

When designing and operating a recirculating system, management plans and systems should be developed to maintain the function of the system under unusual conditions such as disease outbreaks. It is important to understand that biofilter bacteria can be killed by therapeutic agents, such as antibacterial agents and parasiticides, that may be used during disease outbreaks (Heinen et al. 1995). It is also highly important to follow start-up procedures and preconditioning schedules prior to the introduction of fish into recirculating systems.

Effluents

Facilities holding fish will produce wastewaters, and the potential effects of these wastewaters on the receiving ecosystems must be considered. Effluents may be discharged continuously or periodically, may combine with other wastewaters, and may discharge directly to a sewage treatment plant or into other city drainage systems, but ultimately, they will move into a public water body. Most effluents from laboratory wet labs can be safely added to treatment plants or even public water bodies. Regulatory authority and determination of acceptable effluent contributions rests with the USEPA or an EPA-designated authority state or local authorities. Discharge of wastes or pollutants entering waters of the United States requires a National Pollutant Discharge Elimination System (NPDES) permit. The NPDES permit specifies pollutants and concentrations that can be safely discharged. Pollutants not identified in the permit are prohibited from discharge. Such permits are often held by a research institution unless discharge occurs into a sewage treatment facility. In the latter case, the treatment facility would hold the NPDES permit. Individual NPDES permits are required for direct dischargers such as fish farms.

Fish farms are designated by the USEPA as concentrated aquatic animal production

facilities according to their size and the type of fish produced (40 CFR 122.24). Coldwater fish facilities, such as farms and hatcheries for trout and salmon (family Salmonidae) that produce 9,090 kilograms per year of fish or feed 2,272 kilograms feed per month are classified as a concentrated aquatic animal production facilities and need NPDES permits. Warmwater facilities that discharge effluents 30 days per year or produce greater than 45,454 kilograms per year fish also need NPDES permits. Smaller aquaculture facilities may need permits. Researchers conducting tests in aquaculture facilities where an undeclared pollutant, such as a new drug treatment, might be discharged need to contact the USEPA or its designee to determine safety. Failure to secure discharge approval can result in substantial fines and incarceration.

Dangerous Species and Specimens

In addition to the recommendations provided in Section V, researchers holding dangerous species under laboratory conditions should provide special holding units designed to control the specific problem presented by the dangerous animals. As with field studies, individuals working in a laboratory with dangerous species must be provided with training that addresses the specific problems related to each species.

Restraint of Fishes: Anesthetics and Related Chemicals

Prolonged stressful restraint should be avoided. In some cases, utilization of general anesthesia for restraint may be advisable (Mundy and Wilson 1997); however, the benefits of anesthesia and potential effects on data derived from anesthetized fish should be weighed against results obtained from fishes that have not been anesthetized. The full range of potential effects on the subject fish, not just the anesthetic qualities, must be considered. The anesthetic chosen should be one that permits a rapid return to normal physiological and behavioral status (Smith et al. 1999) and is a low-risk compound for humans as well as fish. It should be tested on a small sample of fish prior to use on larger numbers. Anesthetized animals must be kept under observation until appropriate recovery occurs.

The following substances have been used by various researchers (some are controlled substances available only through appropriately licensed sources, such as veterinarians): benzocaine, clove oil, diazepam (valium), sodium pentabarbitol, and tricaine methane sulfonate (MS-222). Hypothermia and exposure to sublethal levels of carbon dioxide (a Low Regulatory Priority drug, see below) have been used in situations where anesthetics were contraindicated. The only anesthetic approved by the FDA for general use on fishes is MS-222, but a 21-day withdrawal period is required.

The FDA allows researchers some degree of choice in the selection and use of drugs, including anesthetics, if the intended purpose of the fish is for research only and the fish will not be consumed or released. Strict interpretation of FDA policies would allow such choice only for approved studies on drugs; however enforcement practices typically allow

greater flexibility. The UFR Committee recommends contact with the FDA Center for Veterinary Medicine to determine current practices and priorities.

The complexities related to FDA drug approvals and the experimental use of drugs in research situations is illustrated by recent actions relative to clove oil. Clove oil or eugenol may not be used in any form on fish that could possibly be consumed by humans, even if the treatment occurs in a laboratory setting. This includes endangered species or species that otherwise may be released into public waters where they would be available for human consumption. The only exception would be under the auspices of an Investigational New Animal Drug (INAD) exemption in which a treatment authorization, including an appropriate withdrawal time, has been obtained from the FDA. (Isoeugenol is a possible substitute for clove oil or eugenol. At this time, there is one publicly disclosed INAD exemption file for the use of isoeugenol as a fish anesthetic and it is held by the USFWS. The product has been given an investigational withdrawal time of 21 days for the dosing regimen being used in the current studies. Studies that could be cited to help demonstrate the safety or effectiveness of isoeugenol in fishes should be coordinated with the USFWS National INAD Office.

Surgical Procedures

Surgical procedures, such as implanting tags and transmitters, or examining gonads have become part of many research plans. Given the aquatic environment in which fishes live, it is impossible to conduct surgical procedures under sterile conditions, even within laboratory settings. Care should be exercised to prevent the introduction of additional infective agents and to minimize the physiological stress of such procedures. Animals subjected to surgical procedures in the laboratory should be observed carefully for at least 72 hours following the surgery.

Administration of Drugs, Vaccines, Hormones, and Other Chemicals

As a general rule, the use and administration of drugs and hormones should be done only in the manner approved by the FDA. The label on the substance describes acceptable uses.

Regulations published in the Code of Federal Regulations allow the use of investigational drugs in a laboratory setting without specific notification to the FDA (21 CFR 511.1[a]). However, these uses are intended for preliminary studies to support the approval of the drug, not for routine clinical use on laboratory animals for another purpose. There is also a provision in the CFR for use of drugs in animals in teaching settings, etc. (21 CFR 201.125). Under this provision, instructors in the fields of pharmacy, chemistry, medicine, law enforcement, research, and analysis are exempt from having to have adequate directions for use. Access to the full citations for the above statements is possible through the Government Printing Office web site.

Further information on drugs for use in research facilities can be obtained at the following links:

Low Regulatory Priority drugs
Drugs in outdoor research facilities
Extra label use
Safe levels of unapproved drugs in aquaculture

The FDA can require researchers to provide complete records of the kinds of drugs used, the amounts used, and the intended purpose for using the drugs; therefore, researchers must keep detailed records of drug purchases, storage, uses, and disposal. Any chemical or other substance that is used to treat a disease condition may be considered to be a drug.

The APHIS approves vaccines and all biologics. Uses must be in accord with those approved by APHIS. Individuals in charge of a research investigation are responsible for using drugs, biologics, and other chemicals in accordance with federal, state, and local regulations and must maintain records to document all uses. It is advisable to establish a client relationship with a veterinarian. Some substances can be administered only by or under the direction of a licensed veterinarian. In addition to their broad expertise in animal health, licensed veterinarians have the authority to prescribe extra-label uses for drugs that may be of value in specific situations and can advise on required tracking, record-keeping, and proper storage of therapeutic agents.

VIII. Storage or Disposition of Experimental Animals

Euthanasia

There are several ways to euthanize fishes humanely. Various regulatory or granting agencies may require specific euthanasia methods and written protocols that demonstrate sufficient attention to humane treatment. In general, it is essential that the procedure be performed quickly. The methods for euthanasia listed by the Royal Society and Universities Federation for Animal Welfare (1987) may be followed.

Pithing, spinal cord dislocation, or decapitation generally are acceptable methods, provided the procedure is performed quickly and accurately. Small fish, less than 10 cm in length, may be euthanized instantly by immersion in liquid nitrogen. Depending on the size of the fish and experimental needs, some form of physical anesthesia, such as hypothermia, may be indicated prior to euthanasia. Cold shock and electrical shock are used commonly by fish processors preparing large numbers of animals for slaughter. Small numbers of fish can be euthanized by exposure to relatively high concentrations of anesthetics such as MS-222; however, the use of MS-222 and other chemical anesthetics

as euthanizing agents has not been approved officially by the FDA.

Euthanasia through simple oxygen deprivation (de-watering) is sometimes practiced during mandated depopulation of production-level facilities; however, this procedure is not recommended for research situations. Stunning with an electroshock followed by rapid decapitation or cold shock are suggested alternatives if large numbers of fish must be euthanized. Selection of euthanasia methods should be done in coordination with the institutional IACUC. Additional information, including the 2000 Report of the AVMA Panel on Euthanasia, can be obtained from the American Veterinary Medical Association (AVMA 2001) .

Marine fish surveys conducted at sea present a special set of conditions with respect to euthanasia. The capture methods tend to collect substantial numbers of specimens at one time. Information on sex, maturity state, and stomach contents may be taken from individual fish that are not dead when processed. Decapitation or pithing of individual fish for otolith removal may be used on such surveys, but these techniques are not suited to processing large numbers of fish. The largest possible portion of the catch must be worked up in the shortest possible time to get the maximum amount of data within the time allotted for each station or sampling event. Euthanasia of individual fish could result in a significant compromise in the amount of data collected. With the exception of certain shark species (subclass Elasmobranchii), or threatened species such as sea turtles (family Chelonidae) and sturgeon (family Acipenseridae), the entire catch may be treated as sampling without replacement. Under such conditions, with constraints of time and the cost of ship time, researchers and agencies should be granted exemptions from standard practices for euthanasia.

Storage, Disposition, or Return to the Wild

Applications for animal use submitted to IACUCs typically require the principal investigator to state where fish or fish carcasses will be located after the study. Whether required or not, plans for disposition of fishes used in experimental studies should be included in study plans. As a general humane practice, it is preferable that fish be released to appropriate sites (when possible and lawful). Such releases must be approved in advance by the fisheries management agency with jurisdictional authority for the proposed receiving site.

Upon completion of studies, wild-caught specimens should be returned to the wild when practical, ecologically appropriate, and approved by the management authority. Nonnative fishes should never be released into the wild by scientists not specifically authorized to do so. Study specimens that cannot be returned to the wild should be euthanized and archived or disposed (ASIH et al. 1987, 1988). As a general rule, field captured fishes should only be released under the following conditions: (a) at the site of the original capture; (b) if their ability to survive in nature has not been irreversibly impaired; (c) where it can be reasonably expected that the released animal will function

normally within the population; (d) where local and seasonal conditions are conducive to survival; and (e) where release is not likely to spread pathogens (ASIH et al. 1987, 1988). Federal, state or local laws may prohibit release of study animals under any circumstances. Study fishes should never be released into water bodies where they are not native nor in violation of any regulation.

Standard operating procedures should be in place for the disposal of study animals that cannot be released. In some instances, study animals may be distributed to colleagues for further study. After proper euthanization and preservation, study animals may be useful as teaching specimens or as voucher specimens in research collections (see Section V). Otherwise, protocols for disposal established by the research institution and local government should be followed. Study animals that contain toxic substances or drugs should never be disposed of in a way that allows these substances to enter the local environment. Incinerating or autoclaving may be required if the fish may be carriers of disease agents that are not indigenous to the area. Large numbers of dead fish may require use of properly managed "mortality pits." These are graves dug into the ground in which fish are placed with periodic application of hydrated lime. The lime raises the pH to lethal levels, destroying pathogenic agents. If the fish contain contaminants that could enter ground waters, the pit must be sealed in an appropriate manner. Eventually the pit should be filled, at which time it should be completely covered with soil. Such pits should be established in locations where they are isolated from natural waters.

IX. Future Revisions

Periodic revisions of these Guidelines are expected. Although the basic philosophies of "the scientific method" are quite constant, specific techniques and procedures for research investigations evolve over time, sometimes quite rapidly. Investigators are encouraged to send new information and constructive criticisms to the officers of the respective societies.

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Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS) of the USDA, 4700 River Road, Unit 43, Riverdale, Maryland 2077-1231, USA.

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XII. Appendix

Summary Guidelines and Checklist

The following checklist provides a "quick reference" of factors that researchers should consider in preparing plans for Institutional Animal Care and Use Committees. This checklist should not serve as a substitute for the more detailed information presented in

the Guidelines but can be used for record-keeping purposes to ensure that plans are complete.

___ (A) Choice of Taxa

___ (B) Number and Choice of Individuals

___ (C) Population and Genetic Considerations

(1) Captive/Domestic Stocks

(2) Wild Stocks

(3) Threatened or Endangered Species

___ (D) Animal Welfare

___ (E) Living Conditions

___ (F) Water Quality

___ (G) Foods, Feeds, and Feeding

___ (H) Health

___ (I) Stress

___ (J) Anesthesia

___ (K) Euthanasia

___ (L) Permits and Regulations