Guidelines for the Use of Fishes in Research

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Table of Contents

Use of Fishes in Research Committee, 2014 vii

Preface ix

Acknowledgments xi

Statement of Purpose xiii

1. Introduction 1

2. General Considerations 3

2.1 Approval of Research Plans by IACUCs 3

2.2 Project Quality Assurance Plans and Standard Operating Procedures 4

2.3 Statistical Design 5

2.4 Mortality as an Experimental Endpoint 6

2.5 Fish Health Management: Control of Pathogens and Parasites 6

3. Statutory Requirements and Regulatory Bodies 9

3.1 International Regulations and Guidelines 9

3.2 Biosecurity 11

3.3 Federal, State, and Local Regulations 12

3.4 Permits and Certificates 14

4. Animal Welfare Considerations 17

4.1 General Considerations 17

4.2 Stress 17

4.2.1 Stages of Stress 18

4.2.2 Measuring and Avoiding Stress 18

4.3 Nociception and Pain 20

5. Field Activities 23

5.1 Habitat and Population Considerations 23

5.2 Field Collections 23

5.2.1 Permits 23

5.2.2 Natural History Collections 24

5.2.3 Representative Samples 24
5.2.4 Collection of Imperiled Species 25
5.2.5 Museum Specimens and Other Preserved Specimens 26
5.3 Live Capture Techniques and Equipment 28
5.4 Field Restraint of Fishes: Sedatives 28
5.4.1 Drugs Approved for Use on Fish 29
5.4.2 Low Regulatory Priority (LRP) Drugs 29
5.4.3 Investigational New Animal Drugs (INAD) 30
5.5 Dangerous Species and Specimens 30
5.6 Handling and Transport 31
5.7 Facilities for Temporary Holding and Maintenance 32
5.8 Field Acclimation 33
5.9 Collection of Blood and Other Tissues 34
6. Marking and Tagging 37
6.1 General Principles 37
6.2 External Tags and Marks 37
6.3 Internal Tags and Marks, and Biotelemetry 38
6.4 Genetic Markers 40
6.5 Stable Isotopes 41
6.6 Fatty Acids 42
7. Laboratory Activities 43
7.1 General Principles 43
7.2 Confinement, Isolation, and Quarantine 43
7.3 Acclimation to Laboratory Conditions 45
7.4 Facilities for Long-Term Housing of Fishes 45
7.5 Density of Animals 47
7.6 Feeds and Feeding 47
7.7 Water Quality 49
7.8 Water Recirculation Units 50
7.9 Effluents and Permits 51

7.10 Dangerous Species and Specimens in Captivity 51
7.11 Restraint of Fishes: Sedatives and Related Chemicals 52
7.12 Surgical Procedures 53
7.13 Administration of Drugs, Biologics, and Other Chemicals 55
7.13.1 Drugs 55
7.13.2 Biologics and Other Chemicals 56
7.13.3 Chemical Facility Anti-Terrorism Standards (CFATS) 56
8. Final Disposition of Experimental Animals 59
8.1 Euthanasia 59
8.2 Storage or Return to Aquatic Habitat 60
9. Future Revisions 61
10. Literature Cited 63
Appendix 85
Brief Checklist for IACUC Readiness 85
List of Low Regulatory Priority Drugs and Consideration for Their Use 86
Appendix Table 1. Low regulatory priority aquaculture drugs, indications, and doses. 87
Appendix Table 2. OIE-notifiable causative disease agents for fish and amphibians. 88
Index of Terms and Acronyms 89
Note on Additional Readings 90

Use of Fishes in Research Committee, 2014

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Preface

The American Fisheries Society (AFS), the American Society of Ichthyologists and Herpetologists (ASIH), and the American Institute of Fishery Research Biologists (AIFRB) are professional societies focused on the scientific understanding and the global protection, conservation, and sustainability of aquatic animals, fishery resources, and aquatic ecosystems. Their policies and standpoints are based primarily on information developed through scientific practices, but they also reflect ethical concerns, including the conservation of the diversity and abundance of fish populations, and respect for life and life processes. Research investigations on fishes, the environments in which they 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 pursuits for all three societies. Further, these societies believe that their members are responsible not only for advancing scientific knowledge and understanding of fish and fisheries but also 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, respect for the forms of life within various ecosystems, and the humane treatment of animals used in research investigations.

Fishes are worthy of experimental and observational research: they are useful indicators of environmental quality and ecological integrity, and their individual adaptations and physiological specializations make them suitable for use as physiological and biomedical models. Further, fishes are economically important through recreational and commercial activities in that they provide an important source of food for humans and other animals and are popular to catch and to observe.

The authors of the Guidelines for the Use of Fishes in Research (referred to hereinafter as the Guidelines) are scientists, have respect for life, and are professionally trained in a multitude of disciplines. The AFS, ASIH, and AIFRB support the intent of the current version of the Guidelines to aid investigators, institutions, and regulatory authorities in addressing responsible, scientifically valid research on fish and fish habitats. The 2004 Guidelines (Use of Fishes in Research Committee 2004) superseded the Guidelines for the Use of Fishes in Field Research (ASIH et al. 1987, 1988) by the inclusion of content relevant to laboratory research. The current version is in answer to the 2004 call for a periodic document review and update, with a similar suggestion noted herein.

The understanding and welfare of animals used in research can be served best by using a multidisciplinary approach in which data and expertise are derived from such disciplines as ecology, behavioral studies, nutrition, genetics, toxicology, chemistry, endocrinology, physiology, anatomy, and fish health. At the same time, understanding that research is

conducted in a variety of human cultural settings is important. Ideally, scientific procedures, analytical methods, data interpretations, and conclusions based on scientific studies should be consistent across all cultures; however, personal belief systems can and do influence concepts regarding which practices and methods are, or are not, consistent with humane treatment of animals. Some members of the 2014 Uses of Fishes in Research (UFR) Committee also served on the committee that revised the 2004 Guidelines (Use of Fishes in Research Committee 2004). The 2004 and 2014 Guidelines not only reflect the scientific expertise of both UFR Committees but also provide a framework for the promotion of scientifically valid research on fish and fish habitats and for research that is conducted in a manner acceptable to the social communities within which the research takes place.

The Guidelines address both field and laboratory research with fishes and will serve as a resource document on topical themes. Specific information in response to United States laws is a focus here, yet these Guidelines can be applied and adapted internationally by investigators working within their own institutional infrastructure with regard to animal care and use committees. Internet pathway links to various Web sites and documents are included; however, such pathways to online media may change. If readers experience difficulty in reaching a specific Web site, contact the AFS Publications Director at alerner@fisheries.org.

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Respectfully,

Jill A. Jenkins, Chair, UFR Committee, 2014

Statement of Purpose

The 2004 and 2014 Guidelines were developed to provide a structure that advances appropriate attention toward valid experimental designs and procedures with aquatic animals while ensuring humane treatment of the experimental subjects. At a practical level, the Guidelines are intended to provide general recommendations on field and laboratory endeavors, such as sampling, holding, and handling fishes; to offer information on administrative matters, including regulations and permits; and to address typical ethical concerns, such as perceptions of pain or discomfort experienced by experimental subjects. These Guidelines must be recognized as *guidelines*. They are not intended to provide detailed instructions but rather to alert investigators to a broad array of topics and concerns to consider prior to initiating study. At a comprehensive level, the principles upon which these Guidelines are based are broadly applicable, and many of the described practices and approaches can be adapted to situations involving other aquatic animal species and conditions.

Understanding the differences between fishes and other vertebrates, especially mammals, is critically important to conducting scientifically sound research with fishes. Disparities in life histories and mortality rates in fishes versus other vertebrates are critical in designing sustainable sampling levels in fish populations. The UFR Committee points out that (1) compared to mammalian populations, adult populations of many fish species persist despite very high natural mortality rates in juvenile stages by virtue of the fact that most species lay thousands or tens of thousands of eggs; (2) because of these mortality patterns, research on fishes, especially field research or research on early life stages, can involve, and often requires, much larger numbers of research subjects than does research on mammals; and (3) the animal handling and husbandry requirements for fishes are fundamentally different from those for mammals and other vertebrates, in general. Policies, regulations, and recommendations developed for research on mammals, birds, reptiles, or even amphibians are frequently inappropriate for research with fishes. The Guidelines also address some of the ethical concerns that motivate guidelines used for research with other vertebrates, while being mindful of the unique physiology and general nature of fishes.

The Guidelines were developed for general use by investigators within the United States; therefore, the roles, responsibilities, and informational needs of Institutional Animal Care and Use Committees (IACUCs) were given specific attention. All United States institutions that use vertebrate animals for research, teaching, research training, and biological testing are required to create an IACUC to oversee and evaluate all aspects of the institution's animal care and use program. Investigators from other nations who read this document may disregard specific references to U.S. state and federal laws and regulations, as their institutional infrastructure and processes may differ from those of an internal committee such as IACUCs. The principles

described herein, however, are applicable to research on fishes regardless of geographic location. Investigators in other nations may benefit by modifying any of the specific provisions pertaining to the United States, thereby adopting guidelines consistent with the laws and regulations of their own government. The UFR Committee urges that the Guidelines be endorsed and adopted (adapted, where necessary) by those state and federal authorities with regulatory responsibilities for fishes, offices with federal oversight (e.g., National Institutes of Health, Office of Laboratory Animal Welfare; http://grants.nih.gov/grants/olaw/olaw.htm) as well as by universities and other institutions and authorities using fishes and aquatic animals within their research and teaching programs.

1. Introduction

Experimental studies using live, intact organisms continue to play an essential role in developing knowledge and better understanding of life processes, life forms, and the environments in which they occur. The enormous evolutionary radiation of fishes comprises at least 27,000 species (Nelson 2006). Fishes exist in a multitude of forms and have many unique physiological, behavioral, and ecological specializations. Fishes occupy a variety of niches in virtually every kind of aquatic habitat. Understanding their biology 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 and terrestrial animals and are an important source of food for other aquatic animals; fish are an important part of aquatic environments and ecological systems; catching and observing fishes are very popular and economically important recreational and commercial activities for millions of people around the world; the unique adaptations and physiological specializations of fish make them especially suitable for use as physiological and biomedical models; and the human endeavor to understand the roles that various organisms play in the earth's ecosystems must include accurate and detailed knowledge of the biology of fishes.

The diversity demonstrated by the 27,000+ species of fishes creates many opportunities for new research, but it also makes the task of developing research protocols that would apply to all species and all circumstances impossible. Instead, broad guidelines building on the most current, scientifically valid information are provided in the Guidelines for interpretation and application by various investigators who frequently are the authority on the species or systems involved in their studies. Ultimate responsibility for the ethical and scientific validity of each study and the methods employed rests with the investigator; however, government agencies, reflecting the beliefs and values of the citizenry and acting on their behalf, often require that investigators follow codes which prescribe acceptable strategies, techniques, facilities, conditions, and post-experimental disposition of animals used in research.

Some individuals have argued that fishes should not be included under laws and policies aimed primarily at mammals and birds; however, the Health Research Extension Act (HREA) of 1985 (Public Law 99-158 1985, http://history.nih.gov/research/downloads/PL99-158.pdf) included fishes within its jurisdiction and responsibilities. Additional information about the HREA of 1985 and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare 2002, http://grants.nih.gov/grants/olaw/references/phspol.htm) may be accessed via the Web sites of

the U.S. Department of Health and Human Services (USDHHS; http://www.hhs.gov/) National Institutes of Health (NIH; http://www.nih.gov/) Office of Extramural Research (OER; http://grants.nih.gov/grants/oer.htm). Fishes are specifically included within the scope of the Guide for the Care and Use of Laboratory Animals—Eighth Edition by the Institute for Laboratory Animal Research (ILAR) (NRC 2011, http://www.aaalac.org/resources/theguide.cfm), Division on Earth and Life Studies, National Research Council of the National Academies. The laws and other guides, such as the ILAR guidelines (NRC 2011) provide general material addressing fishes or other poikilotherms. The ILAR guidelines specifically call for the development of detailed information by knowledgeable groups. Generally, scientific societies with expertise on particular classes of vertebrates are considered to be the most appropriate sources for the supplemental information needed to implement existing policies.

The pre-2004 versions of these Guidelines, Guidelines for the Use of Fishes in Field Research, were developed and jointly published by the American Society of Ichthyologists and Herpetologists (ASIH), American Fisheries Society (AFS), and American Institute of Fishery Research Biologists (AIFRB) (ASIH et al. 1987, 1988). The 1987 and 1988 Guidelines emphasized field research, and the 2004 Guidelines added material on laboratory research with fishes. The 2004 Guidelines were thus developed in response to the Public Health Service (PHS) expanding their definition of "animals" to include live vertebrates used in research or intended to be used in research (Public Law 99-158). The 2014 Guidelines expands the 2004 Guidelines by providing updates with new knowledge and information brought forward over this past decade. A checklist to assist investigators preparing Institutional Animal Care and Use Committee (IACUC) applications remains in these 2014 Guidelines (see Appendix).

2. 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 introduces concepts and procedures that can be adapted to the situation and circumstances for each investigator.

Research studies should have well-understood and justifiable objectives that address, within the context of the research discipline, basic needs for knowledge and understanding of the world in which we live and the particular resource under consideration. In cases where the biology of fishes proposed for study is well known, a study hypothesis should be articulated so that the use of fishes is focused on addressing that hypothesis. If the biology of a fish species is not well known, general sampling and other observational uses of fishes (i.e., not hypothesis-driven) may be warranted to guide the development of a good study hypothesis (see section 5.2 Field Collections). Research quality relies on the carefully communicated questions that can be addressed by scientific methods and on the development of research procedures that are quality controlled, publishable, and repeatable. Guidelines have been published for the improved reporting on animal research and for increased reproducibility (Kilkenny et al. 2010; see ARRIVE Guidelines, http://www.nc3rs.org.uk/page.asp?id=1357; see section 3.1 International Regulations and Guidelines).

The validity of research results is affected by the experimental design, the analytical procedures employed, and the quality and health status of the experimental subjects. The quality and appropriateness of the fishes used, both the species and the individuals, can seriously influence results and conclusions, thereby having dramatic effects on the number of animals needed and the number of times that the study is 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 prior to 1985, when requirements that research proposals obtain the approval of an IACUC were imposed (Public Law 99-158 1985, http://history.nih.gov/research/downloads/PL99-158.pdf). 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 are applicable to investigators around the globe.

2.1 Approval of Research Plans by IACUCs

When approval by an IACUC is required, investigators must prepare written statements or requests for animal use that ensure that all applications, proposals, and actual research will meet certain basic requirements (Silverman et al. 2007). Safeguards include limiting unneeded replications of research, use of appropriate species and number of animals, an adequate experimental design, and method of euthanasia or disposition of the animals after the completion

of the study. Investigators must be familiar with the species to be studied, or with 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, restraint, or other changes in environmental conditions that may be applicable to the particular study. Copies of IACUC-approved study plans are maintained by the IACUC from the sponsoring facility, as well as by the individual investigator.

Post-approval monitoring of protocols by the IACUC is required by federal laws, regulations, and policies; allows for the modification of research procedures/protocols; and ensures the well-being of the animals.

2.2 Project Quality Assurance Plans and Standard Operating Procedures

Project Quality Assurance Plans (PQAPs) and standard operating procedures (SOPs), while not required by many universities or nongovernmental institutions, are nevertheless recommended as useful tools for maintaining overall research quality. PQAPs and SOPs usually are required if research data and conclusions will be submitted to certain regulatory agencies, such as the U.S. Food and Drug Administration (FDA, http://www.fda.gov/) or the U.S. Environmental Protection Agency (EPA, http://epa.gov/).

A PQAP should include a description of the project design and approach to the problem, a statement of anticipated deliverables, and a description of how the data will be reported (for instance, as individual measurements or in a reduced form). The PQAP documents the principles, policies, organizational responsibilities, objectives, implementation actions, and accountability procedures that will ensure an appropriate quality process throughout a research project. Emergency contacts and contingency plans for animal care may be included in a PQAP. Importantly, the PQAP should describe actions that will be used to control/verify experimental procedures and ensure that results obtained are representative of the experimental parameters being evaluated. The PQAP should identify how unauthorized laboratory personnel or research project areas will be controlled and biosecurity maintained. For research involving field sampling, the PQAP should include a sampling plan outlining the methods, equipment, and procedures, as well as safety procedures for personnel. For laboratory research, sample preparation and analytical methods should be specified. SOPs for routine, specialized, or unpublished methods should be identified. Data quality objectives should be identified for each measured parameter, and quality control procedures to be used, whether in the field or under laboratory conditions, should be stated. Corrective actions should be specified should problems arise. Finally, a quality assurance (QA) report should be generated at the conclusion of the study. This report should include a summary of the PQAP, results of technical systems and performance evaluation audits, corrective actions taken and results of those actions, data quality assessments (precision, accuracy, representativeness, completeness, comparability, and reporting

limits), a discussion of whether the QA objectives were met, and limitations on use of data collected during the research.

SOPs document routine or repetitive technical activities, or those that occur occasionally yet need to be followed in the exact fashion as the previous time. Ideally, SOPs ensure that work is done correctly the first time, thereby reducing unnecessary repetition and costs. If research data are generated in a setting where the same procedures are used yet personnel change with time, SOPs help maintain consistency. Some institutions require SOPs for assuring that job applicants can perform the needed tasks. SOPs 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 laboratories or institutions. Examples of specific technical tasks for which SOPs are useful in conducting research with fishes include blood sampling, vaccination protocols, procedures for electrofishing, preparation of solutions for use as sedatives, and techniques for collecting meristic data. Policies requiring adherence to SOPs and PQAPs in research protocols are to allow for the development of new procedures and for the revision or expansion of established procedures whereby new study techniques are developed. Additional information on SOPs and PQAPs is available in guidance documents from the EPA (EPA 2007, Guidance for Preparing Standard Operating Procedures [SOPs], http://www.epa.gov/quality/qs-docs/g6-final.pdf, or EPA 2002, Guidance for Quality Assurance Project Plans, http://www.epa.gov/quality/qs-docs/g5-final.pdf) or the FDA (various questions and answers regarding quality assurance and SOPs are included in FDA 2007, Guidance for Industry—Good Laboratory Practices Questions and Answers, http://www.fda.gov/downloads/ICECI/EnforcementActions/BioresearchMonitoring/UCM133748.pdf) and from various manuals or texts that address quality assurance. Further, the U.S. Fish and Wildlife Service (USFWS, http://www.fws.gov/) Aquatic Animal Drug Approval Partnership (AADAP) Program (http://www.fws.gov/fisheries/aadap/home.htm) has online SOPs (http://www.fws.gov/fisheries/aadap/AADAP_Protocols_and_SOPs.htm).

2.3 Statistical Design

Inherent in the use of animals in research is the responsibility for efficient, effective design of experimental studies, wherein healthy animals without abnormal physiognomies and behaviors will facilitate study results, and for humane treatment of experimental subjects (Klontz and Smith 1968; Snieszko 1974; Klontz 1995; NRC 2011). Study objectives should be clearly stated, and explanations should be provided on the need for the type and quantity of data to be collected, as well as what will constitute an end to the experiment.

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. The life stage of the fish used in each study will also affect the numbers needed.

Studies of early life stages may 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 ensure 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). Statistical power analysis can improve designs of experiments (Peterman 1990). Conducting statistical power analyses ensures the development of study designs that have the appropriate statistical power to accomplish research objectives.

2.4 Mortality as an Experimental Endpoint

In laboratory studies, experimental endpoints, other than death of the experimental subjects, should be developed unless mortality 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 (e.g., short-term, limited mortality studies may be used to develop experimental limits for subsequent sublethal studies), and (2) mortality data are required, or at least preferred, by a sponsoring agency to provide a basis for criteria development as part of a regulatory process. Studies that require mortality endpoints include, but are not limited to, those concerning the effects of pathogens and parasites, toxicological research, and physiological tolerance.

2.5 Fish Health Management: Control of Pathogens and Parasites

In laboratory studies involving fishes, healthy subjects are prerequisites for reliable data (Jenkins 2011a), unless an infectious disease is part of the experimental protocol. Fish used in research must be free of any notable microbial presence that could indicate a diseased condition. Fish free from infectious fish pathogens generally will be satisfactory; however, an unrecognized disease condition, even at chronic or nonlethal levels, can seriously confound research results (Lawrence et al. 2012). The source of fish used in research will, in general, influence their health status. Fish raised in captivity have a level of health oversight that will not occur in wild-caught fish. When inquiring about the health status of fish at a culture facility, the researcher can request specific information including any available fish health inspection reports. When fish are brought into a laboratory setting from the wild, the researcher should expect that microorganisms are present. If no disease symptoms are apparent, this is no guarantee that these wild-caught fish are free from problematic disease organisms. Once those fish are in a laboratory setting, the culture conditions and associated stressors will be very different from those in the natural environment, whereby an active disease event can develop. Many laboratories will administer formalin baths to newly arrived fish during an acclimation period (see section 7.3 Acclimation to Laboratory Conditions). The goal is to eliminate external protozoa and monogeneans from the

fish. It is not recommended that fish be routinely administered antibiotics for bacterial pathogens as this practice may lead to the development of antibiotic resistant bacteria.

Any fish that die in the laboratory setting should undergo a proper diagnostic evaluation by an individual with expertise in fish health. Determining the cause of such mortalities will greatly aid the development of health management protocols for a facility. For laboratory colonies of fish that are maintained on a long-term basis, a surveillance program may be established whereby a limited number of fish are selected at regular intervals for diagnostic evaluations to determine if any problematic pathogens are present. As with the performance of diagnostic evaluations on mortalities, data gained through surveillance efforts will provide valuable information regarding if pathogens are present in a colony so that appropriate management practices can be put in place to limit any impacts. The researcher should consult with an individual with expertise in fish health and/or the veterinarian with responsibility for health management at their facility for further information.

In fact, in both captive-reared and wild-caught fishes, the investigator may expect to find various infectious organisms. The presence of such infectious organisms may not cause disease or prevent the use of the host fishes 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 fishes to be "pathogen 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 any fish health issues in a manner that provides for the health and well-being of the fish and also supports the research.

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 fishes are treated for a disease with a therapeutic compound prior to study initiation, they should not be used 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 in the analyses of data derived from that study.

If experimental fishes are to be treated for a disease, FDA-approved drugs should be used and current FDA regulations followed (Code of Federal Regulations [CFR] 2012), 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 EPA, 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, the UFR Committee believes that considerable caution should be exercised in the use of any drug that has not received FDA approval. There are some compounds which are not currently approved but which can be legally accessed for use in fish (e.g., products included in the Index of Legally Marketed Unapproved New Animal Drugs for Minor Species, drugs used under Investigational New Animal Drug [INAD] exemptions). Additionally, unapproved drugs of "low regulatory priority" (LRP) can be used, provided that some conditions are met (FDA 2011; see Appendix Table 1). Please see section 7.13 Administration of Drugs, Biologics, and Other Chemicals for additional details on the options regarding drug use in fishes. A list of the substances that seem effective but are as yet unapproved are not included here because of the danger that such an inclusion could be misconstrued as endorsement of unapproved drugs. Fishes treated with substances that have not been approved by the FDA must not be released nor consumed.

Complete records of all disease treatments must be maintained because FDA inspectors may order a review of these files. Approved therapeutic compounds have information sheets or labels that specify guidance on the use of that substance with the fish species. 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 ultimately receive their concurrence. If the fishes may eventually be released or if they could become food items 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 (http://www.fda.gov/) and the EPA (http://www.epa.gov/).

3. Statutory Requirements and Regulatory Bodies

The investigator must have knowledge of all regulations pertaining to the animals under study, as well as to biosecurity issues, 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.

3.1 International Regulations and Guidelines

Investigators working outside of the United States should comply with all wildlife regulations of the country in which the research is being performed. Work with many species is regulated by provisions of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; http://www.cites.org/), an international agreement with an aim to ensure that international trade in specimens of wild animals and plants does not threaten their survival. CITES promotes that "wild fauna and flora in their many beautiful and varied forms are an irreplaceable part of the natural systems of the earth which must be protected for this and the generations to come" (CITES 1979, http://www.cites.org/eng/disc/text.php). Trades range from live animals to a vast array of wildlife products, including sturgeon caviar. The CITES Web site includes a database of species and maps. The text of the Convention was finalized in Washington, D.C., in 1973 following a 1963 resolution adopted by members of World Conservation Union. Member countries (Parties) adhere voluntarily, and when they have "joined" CITES, they are legally bound to implement the Convention. The regulations of the Convention, however, do not replace national laws; rather, they provide a framework to be respected by each Party. Each Party then adopts its own domestic legislation to ensure that CITES is implemented at the national level.

International trade in animals and animal products calls for regulations designed to prevent the spread of transmissible diseases to individual animals, between groups of animals, and to humans (Jenkins 2011b). Disease risks should be assessed and precautions taken to minimize risks before wildlife is translocated (Cunningham 1996) (see sections 3.2 Biosecurity and 7.2 Confinement, Isolation, and Quarantine). The World Organisation for Animal Health (formerly known as the Office International des Epizooties; OIE, http://www.oie.int/), created in 1924 and headquartered in Paris, has been a leader in defining international health standards for animals, with a current membership of nearly 200 countries. Focus includes global animal health and disease and the dissemination of veterinary science information. They publish two Codes (terrestrial and aquatic) and two Manuals (terrestrial and aquatic). The Codes aim to assure the sanitary safety of international trade in animals and their products. The Aquatic Animal Health Code (OIE 2012a, http://www.oie.int/en/international-standard-setting/aquatic-code/) addresses animal health and zoonoses, animal production, food safety, and animal welfare. The Manual of Diagnostic Tests for Aquatic Animals (OIE 2013, http://www.oie.int/en/international-standard-

setting/aquatic-manual/access-online/) describes internationally accepted laboratory techniques. (See Appendix Table 2 for a list of OIE-notifiable diseases in fish and amphibians.)

The Organization for Economic Cooperation and Development (OECD, http://www.oecd.org/), headquartered in Paris, has 34 member countries and promotes policies that intend to improve economic and social well-being. Publications through OECD address fish and fisheries policies with topics including fisheries globalization and toxicological testing guidelines. These publications take into account sustainable communities and animal welfare concerns. Annual reports, newsletters, working papers, guidelines, best practices, and legal instruments can be viewed through the OECD Web site.

Countries may participate in international fisheries forums through such organizations as the Food and Agriculture Organization of the United Nations (FAO, http://www.fao.org), the Ocean and Fisheries Working Group of the Asia-Pacific Economic Cooperation (APEC, http://www.apec.org), and the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR, http://www.ccamlr.org). Guidelines written for specific countries may be researched through contact with such international authorities.

In the European Union (EU), the 27 countries' activities and partnerships are based on the rule of law, whereby treaties and binding agreements are upheld. For the care and use of live animals for scientific purposes, the European Commission (the executive body of the EU) has promoted the "3R" principles of replacement, reduction, and refinement. The "3R" principles intend to facilitate alternative approaches, reduce animal use, and refine the animal procedures (European Union 2010; see section 2. General Considerations). This legislation aims at eliminating disparities among member states' laws, regulations, and administrative provisions regarding the protection of animals used for experimental and other scientific purposes, thereby lessening barriers to trade involving products and substances resulting from the research. Specific guidelines on vertebrates are provided. Article 49 states that each member state is to establish a national committee for the protection of animals used for scientific purposes. The committees are to provide advice on the acquisition, breeding, accommodation, care, and use of animals in scientific procedures, thereby ensuring the use and sharing of best practices (European Union 2010). For example, amendments of the Hungarian Act XXVIII of 1998 on the Protection and Humane Treatment of Animals regulate the use of animals for scientific research (Act XXVIII 1998; Government Decree No. 243/1998 (XII. 31.) 1998; Joint Decree of the Ministry of Agriculture and Regional Development, Ministry for the Environment, and the Ministry of Economics No. 36/1999 (IV.2) 1999). Documents within EU law are available online (http://eur-lex.europa.eu/). In the United Kingdom, the updated Animal Welfare Act 2006 (Department for Environment, Food and Rural Affairs [Defra] 2006) encompasses all vertebrates, including fishes. Under Item 59 of the Act, angling, which pertains to catching and

landing fish, is excluded; however, responsibilities for operating a fishery and for the welfare of the fishes contained therein are not excluded (Defra 2006). The Centre for Environment, Fisheries and Aquaculture Science (Cefas, http://www.cefas.defra.gov.uk/), an executive agency of Defra, works with scientific entities to deliver science-based recommendations, conducts research, and facilitates international relationships. An additional resource is the Fish Veterinary Society (FVS, http://www.fishvetsociety.org.uk/), a forum for veterinary surgeons, fish health professionals, and veterinary students. The FVS promotes fish care and health management for multiple settings, including those for ornamentals.

In Australia, the Department of Agriculture (http://www.daff.gov.au/) plays an important role in promoting the biological, economic, and social sustainability of Australian fisheries and provides a multitude of services and resources for the fisheries communities, including biosecurity concerns of importing and exporting countries. The Fisheries Research and Development Corporation (FRDC, http://frdc.com.au/research/Pages/default.aspx) is a partnership between the government and the fishing and aquaculture industries for Australian research partners. The Australian Fisheries Management Authority (AFMA, http://www.afma.gov.au/) establishes research priorities (http://afma.gov.au/resource-centre/reesearch/research/), the legislation and policy for which were defined mostly in the 1990s (http://www.afma.gov.au/about-us/legislation-and-policy/).

In Canada, the Canadian Council on Animal Care (CCAC, http://ccac.ca/) oversees the ethical use of animals in science. Governed by a council of representatives from more than 20 national organizations, the CCAC is a "quasi-regulatory" body that sets standards through guideline documents and policy statements on animal use in science. The CCAC guidelines on the care and use of fish (CCAC 2005, http://ccac.ca/Documents/Standards/Guidelines/Fish.pdf) were developed in response to the increased use of fishes as experimental subjects. The Canadian Food Inspection Agency (CFIA, http://www.inspection.gc.ca) and Fisheries and Oceans Canada (http://www.dfo-mpo.gc.ca) are responsible for developing containment guidelines for fish pathogens.

3.2 Biosecurity

Biosecurity can be defined in many ways (Falk et al. 2011); it is the process of taking precautions to minimize the risk of introduction and spread of infectious organisms into or among populations. The FAO defines biosecurity as a strategic and integrated approach that encompasses the policy and regulatory frameworks that analyze and manage risks in the sectors of food safety, animal life and health, and plant life and health, including associated environmental risk. FAO declared that "biosecurity covers the introduction of plant pests, animal pests and diseases, and zoonoses, the introduction and release of genetically modified organisms (GMOs) and their products, and the introduction and management of invasive alien species and genotypes" (FAO 2013, http://www.fao.org/biosecurity/). Disease occurrence is

dependent on the health of the animals, the condition of the environment, and the presence of a pathogen at levels sufficient to negatively affect health (see section 7.2 Confinement, Isolation, and Quarantine). The term "biosecurity" is also relevant to environmental biodiversity. Generally, regulations, appropriate permits (see section 3.4 Permits and Certificates), and other specific concerns regarding biosecurity within a country are addressed within the guideline documents mentioned herein. New Zealand, by virtue of its unique geographic isolation and economic agricultural base, has a plethora of legislation dealing with biosecurity issues (Biosecurity Act 1993).

Various circumstances hold biosecurity as a concern with regard to working with fish. These include state, regional, national, and international transfers of fish and fish products, aquaculture production, and the ornamental industry. Implementation of biosecurity at the global level and organizations working therein were delineated by Scarfe (2003). Biosecurity is a dynamic discipline because of advances in diagnostic technologies and knowledge about epidemiology and pathogenesis. When considering cryopreserved gametes and early life stages of aquatic species, biosecurity practices enable artificial spawning methods to deliver genetics safely. Issues involve disease transmission, introduction of exotic species, genetic consequences for target species, and genetic consequences for ecosystems (Tiersch and Jenkins 2003). Microorganisms (see section 5.9 Collection of Blood and Other Tissues) in archival samples can jeopardize valuable germplasm resources by lowering cell quality (Jenkins 2011b). Pathogen control strategies are a concern for small fish models used as biomedical models, especially Zebrafish *Brachydanio rerio* (also known as zebra danio *Danio rerio*), Japanese Medaka *Orzyias latipes*, and species of the genus *Xiphophorus* (Lawrence et al. 2012). Contemporary international and national regulatory frameworks, treaties, partnerships, and agreements addressing the transfer of aquatic animals and aquaculture products can be adapted as mechanisms for the oversight of unique temporal and geographic biosecurity issues inherent with the circumstance. Some countries may already have regulations, which are likely aligned with OIE recommendations and EU requirements, for the activities of mammalian artificial insemination industries. Additionally, core institutional biosecurity tenants may be necessary in achieving compliance with international regulations.

3.3 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 U.S. Department of Health and Human Services (HHS; http://www.hhs.gov/) and the U.S. Department of Agriculture (USDA; http://www.usda.gov/wps/portal/usda/usdahome). If endangered, threatened, or candidate species for listing are involved, the U.S. Department of the Interior (http://www.fws.gov/endangered/) or the U.S. Department of Commerce (http://www.nmfs.noaa.gov/pr/) has additional authorities. Authority for each Department is found in specific Acts of Congress. Legislative mandate for the Public Health Service (PHS; http://www.usphs.gov/) policy for use of animals in research is provided by the Health Research

Extension Act of 1985 (Public Law 99-158 1985, http://history.nih.gov/research/downloads/PL99-158.pdf). This Act charged the Secretary of Health and Human Services with the responsibility of establishing guidelines for proper care and treatment of animals used in research and for organizing and operating animal care committees. 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 PHS Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare 2002, http://grants.nih.gov/grants/olaw/references/phspolicylabanimals.pdf), promulgated in 1985, includes the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. This provides a framework for conducting research in accordance with the PHS policy. The PHS requires institutions to use the Guide for the Care and Use of Laboratory Animals (NRC 2011, http://www.aaalac.org/resources/theguide.cfm).

The legislative mandate for animal welfare, as dictated by the USDA, is contained in the Animal Welfare Act (AWA), as amended (7 USC, 2131–2156). The AWA Amendments of 1970 (Public Law 91-579 1970, http://awic.nal.usda.gov/government-and-professional-resources/federal-laws/animal-welfare-act) expanded the list of animals 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 AWA, investigators should be familiar with the general content and intent of the AWA. The complete AWA, including all amendments (1970, 1976, 1985, and 1990) following the 1966 enactment, can be found in United States Code (2012). The USDA regulations implementing the AWA can be found in CFR (2013). A compilation of information sources related to fish welfare is available from the USDA (Erickson 2003, http://www.nal.usda.gov/awic/pubs/Fishwelfare/fishwelfare.pdf).

The USDA Animal Welfare Information Center (http://awic.nal.usda.gov/) is mandated by the AWA to provide information for improved animal care and use in research, testing, teaching, and exhibition. The establishment of an IACUC is introduced with a description of its roles, composition, and responsibilities to the USDA (Office of the Deputy Administrator, National Program Staff 2002, http://www.afm.ars.usda.gov/ppweb/PDF/130-04.pdf). A compilation of information sources relevant for biomedical research and amphibian, fish, and reptilian animal models is available (Crawford et al. 2001, http://www.nal.usda.gov/awic/pubs/amphib.htm). Principles and procedures that govern research, testing, and teaching activities involving laboratory animals in the Department of Veterans Affairs is available (Veterans Health Administration 2011, http://www.va.gov/vhapublications/ViewPublication.asp?pub_ID=2464). The Ornithological Council has online Guidelines to the use of wild birds in research (Fair et al. 2010, http://www.nmnh.si.edu/BIRDNET/guide/index.html), and the American Society of

Mammalogists has one for the use of wild mammals in research (Sikes et al. 2011, http://www.mammalsociety.org/uploads/committee_files/Sikes%20et%20al%202011.pdf).

The Food Security Act of 1985, subtitle F–Animal Welfare (Public Law 99-198 1985, http://awic.nal.usda.gov/public-law-99-198-food-security-act-1985-subtitle-f-animal-welfare), also called the Improved Standards for Laboratory Animals Act, suggests minimum requirements in specifics such as sanitation, housing, and ventilation. The Act specifies that procedures that may cause distress (see section 4. Animal Welfare Considerations) are to be minimized in experimental procedures and that alternatives to such procedures are to be considered by the principal investigator. It also specifies elimination or minimization of unnecessary duplication of experiments on animals to help allay public concern for laboratory animal care and treatment.

States and tribal authorities may have specific legislative statutes that empower them to regulate the use of animals in research. Typically, such regulations may be found in the laws pertaining to natural resources, health, and agricultural use of fishes and wildlife and are available through the appropriate state government agency. Interstate transport of fishes, and in some situations intrastate transport, is regulated at the state level. Investigators are urged to determine which laws may apply to their research conduct. Local authorities rarely oversee the conduct of research; however, investigators should recognize that local regulations relative to the conduct of their studies may exist.

3.4 Permits and Certificates

In addition to government regulations pertaining to the conduct of research, permission typically is required for the transport of animals across state or international boundaries (see section 3.2 Biosecurity) and the discharge of effluents from a confined operation (see section 7.9 Effluents and Permits). In the United States, authority for interstate transport of fishes is usually under the jurisdiction of the states' fish and game agencies, just as a "take" by angling or other means requires a permit (see section 5.2 Field Collections). Documentation may be required for transport or shipment across state lines, for receipt of shipment, and sometimes for intrastate transport. Permits are typically required for injurious or invasive species. The USFWS is a resource for importers and exporters (http://www.fws.gov/le/ImpExp/Info_Importers_Exporters.htm). Permits are required to document that the laws (http://www.fws.gov/permits/ltr/ltr.html), Federal Register documents (http://www.fws.gov/permits/FederalRegister/FederalRegister.html), and treaties (http://www.fws.gov/laws/lawsdigest/treaty.html) are being adequately followed in order to help conserve protected resources. Many of these are directly applicable to fisheries investigators, including provisions of CITES, the Northwest Atlantic Fisheries Treaty (International Convention for the Northwest Atlantic Fisheries [ICNAF] 1 U.S.T. 477; T.I.A.S. 2089), and the Great Lakes Treaty (Convention on Great Lakes Fisheries between the United States and

Canada; 6 U.S.T. 2836; T.I.A.S. 3326) (information on these and other treaties is available at http://www.fws.gov/laws/lawsdigest/treaty.html).

In certain cases, jurisdiction over interstate and international movement of fishes may be under the Animal and Plant Health Inspection Service (APHIS, http://www.aphis.usda.gov/) of the USDA. 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. Guidelines for addressing importation of live and dead fish, as well as gametes, are found in Title 50 (Wildlife and Fisheries) Part 16 of the CFR 2008 (http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=1&SID=7a0d8ba4c403a707edcd008028e3e519&ty=HTML&h=L&n=50y1.0.1.2.10&r=PART). The Lacey Act (United States Code 2010; http://www.gpo.gov/fdsys/pkg/USCODE-2010-title16/pdf/USCODE-2010-title16-chap53-sec3371.pdf) combats trafficking in "illegal" wildlife, fish, and plants. The law prohibits the transportation of illegally captured or prohibited animals across state lines and addresses potential problems caused by the introduction of nonnative animal species. With regard to food commodities, those seeking to import fish/aquaculture products and live/raw shellfish from the EU need to consult the National Oceanic and Atmospheric Administration Seafood Inspection Program of the Department of Commerce (see http://www.seafood.nmfs.noaa.gov/).

4. Animal Welfare Considerations

4.1 General Considerations

Research involving living animals, including fishes, must be based on experimental designs and animal care practices that can lead to scientifically valid results. Fishes are acutely sensitive to stress (e.g., Barton and Iwama 1991), and responses may include changes in behavior (e.g., Martins et al. 2012), reduced growth, changes in osmotic status, suppressed immune systems (with consequent disease onset), and altered reproductive capacity (Iwama et al. 2006; Schreck et al. 2001; Schreck 2010). Accordingly, unless the experimental objectives require actions or conditions designed to test responses to stress, fishes should be maintained, handled, and tested under conditions that will not create such responses. The Guidelines addresses the conduct of scientific research and focuses on established facts and the processes through which knowledge is developed. Research plans submitted to IACUCs should address animal care considerations, in addition to the details of research goals, objectives, and procedures. The extent to which IACUCs incorporate personal values concerning animal welfare into their institutional guidelines is determined within each institution.

4.2 Stress

The study of stress has focused on how animals have evolved physiological and behavioral mechanisms to address the challenges of changing environmental conditions and then to permit them to maintain homeostasis, or self-sustaining balance. The set of environmental variables (conditions) best suited for the well-being of each species typically encompasses a specific range for each factor and species (see section 5.7 Facilities for Temporary Holding and Maintenance), as stress responses are species-specific (Schreck 2010). Accordingly, when fishes are maintained within these ranges, a state of homeostatic balance is expected. Deviations from homeostasis characterize a stress response. While many definitions for stress have been proposed, we employ the definition of Schreck (2000) and 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 attempt to reestablish homeostasis via a process known as "allostasis regulation in which they adjust their physiological function to re-establish a dynamic balance" (Sterling and Eyer 1988). While allostasis is generally adaptive because it helps keep animals alive in the face of a short-term stressor(s), it can be maladaptive over the long term and have negative consequences on growth, reproduction, and immunological health (Schreck 2010). Accordingly, investigators need to understand those factors that might cause stress in their experimental animal(s), the potential consequences, and how stress might be avoided by optimizing experimental conditions.

Each investigator and the IACUC should understand the conditions that minimize stress for the species in question. Extrapolation between taxa, however, must be avoided because differences exist among species (Schreck 2010). 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, investigators should be aware of APHIS policy (i.e., Policy 11, USDA 2011, http://www.aphis.usda.gov/animal_welfare/policy.php?policy=11).

4.2.1 Stages of Stress

Stress responses are elicited after a fish detects a threat. Recognizing and understanding the three stages of stress is important. Each warrants consideration in the design of animal care protocols:

Stage 1. Primary stress responses vary among species but are characterized by immediate neuroendocrine responses including catecholamine and corticosteroid release and can be quantified by measuring blood hormones. Sometimes behavioral changes accompany these endocrine responses that help the animal cope with the stressor and, in and of themselves, have few consequences to health.

Stage 2. The secondary stage of a stress response is characterized by changes in blood and tissue function evoked by the primary response. Secondary stress typically occurs within minutes of the primary response and is characterized by increased blood glucose and heart rate, diuresis, alteration of leukocyte count, altered osmolyte balance, and behavioral changes (see section 5.6 Handling and Transport). Although these responses can have short-term positive effects, many also are negative, so they should be avoided when possible. They can be evaluated through the study of extracted blood (see section 5.9 Collection of Blood and Other Tissues).

Stage 3. Tertiary stress responses are associated with long-term exposure and negatively affect the well-being of the organism. Effects associated with tertiary stress include decreased growth, propensity to contract disease, and decreased reproductive function (Selye 1976; Schreck et al. 2001; Iwama et al. 2006; see sections 5.8 Field Acclimation and 7.3 Acclimation to Laboratory Conditions). The best way to avoid a tertiary stress response is to care for animals so as to minimize stress responses.

4.2.2 Measuring and Avoiding Stress

While the nature of stress is insidious, it also tends to be polymorphic, changing with time and taking different forms in different species at different stages in their lives. It is rarely feasible to measure changes in blood hormones to assess primary or secondary stress; therefore, investigators are advised to design experiments that avoid stress unless the purposes of the research require measurements of stress indicators. Important indicators of a lack of stress are persistence of normal behavioral activity and propensity to feed and grow. Careful experimental design and planning can ensure study results 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 investigators to select species for experiments whose optimal holding conditions are known and can be recreated in the laboratory. Specific factors to consider include (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, (9) shelter, (10) stocking density, and (11) size of tank relative to body size and activity rate. Other variables, such as fish density or the presence or absence of tank covers, may be important. Species that are known as reliable laboratory models (e.g., Zebrafish or Japanese Medaka) or that are commonly used in fish culture (e.g., Channel Catfish *Ictalurus punctatus* or Rainbow Trout *Oncorhynchus mykiss*) might be selected whenever such a choice is compatible with research objectives.

In addition to the aforementioned factors that are associated with long-term maintenance, additional considerations apply when fishes are handled or subjected to various experimental manipulations.

- Handling should be minimized. Merely catching fish in nets can induce release of stress hormones, such as cortisol, within one minute. Fishes should be given time to recover from handling prior to use in experiments. The amount of recovery time needed may vary with species and conditions; therefore, preliminary tests would help to establish the appropriate recovery period.
- Effects of stressors can be reduced through the use of sedatives or by adding environmental salts to the holding water to reduce osmotic and related stress. (Note that marine fishes, due to their osmoregulatory requirements, can be an exception.) The specific salts and concentrations will vary depending on each fish species and environmental conditions. Sedatives themselves, however, can evoke physiological stress responses (Trushenski et al. 2012a), so they should be employed cautiously and in accordance with established guidelines.
- Environmental conditions from which fish originated, or are held, should not be changed rapidly. This is especially true for temperature conditions. An instantaneous change of 2°C in water temperature generally is not lethal, but it can cause detectable stress responses. Tolerable changes depend on the species, the life history stage, previous thermal history, and the initial holding conditions. Effects due to previous thermal history have been detected for as long as a month posttreatment. Rapid, substantial changes in water quality also should be avoided (see section 7.7 Water Quality).
- Fish densities should be appropriate. Fish which live in shoals should be kept as groups but not in such large groups that they are crowded and compete for food and space or degrade water quality.

- Considerable time to recover from disturbances should be allowed, if compatible with study design. At least 1 week of recovery is preferable, and 24 hours should be considered a minimum even though recovery times are not absolutely known. Although physiological responses may return to prestress conditions more quickly, the fish may be abnormally sensitive to subsequent disturbances for longer periods of time. Resumption of normal feeding and shoaling activity can usually be a good measure of recovery.

As experienced fish culturists and investigators have learned, the critically important axiom for successful maintenance of fishes in captivity is "know your fish." Inexperienced investigators are advised to work closely with experienced personnel 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 reviews (Bonga 1997; Barton 2000).

4.3 Nociception and Pain

Pain is fundamentally a psychological state of the conscious mind. The definition of pain provided by the International Association for the Study of Pain (IASP, http://www.iasp-pain.org/) is widely accepted by the scientific and medical communities. Pain is defined and described by the IASP as (1) "…an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such tissue damage," (2) "…always subjective," and (3) "…sometimes reported in the absence of tissue damage" (IASP 2011, http://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698&navItemNumber=576#Pain). This scientific organization explains that a definition of pain must avoid connecting it to an external eliciting stimulus (Wall 1999; IASP 2011). Scientific difficulty arises when the definition is applied to animals for which the psychological experience of the conscious mind cannot be objectively discerned. Scientists attempting to determine whether fish feel pain have thus developed surrogate metrics which, to date, have shortcomings. Overall, the weight of evidence in the fish species studied indicates that the experience of pain in mammals is not experienced in fish.

To better understand the scientific merits of research articles on welfare-related aspects of aquatic animal physiology, some biases and limitations have recently been elucidated (Rose 2007; Browman and Skiftesvik 2011; Rose et al. 2014). Much of the confusion associated with pain research in animals, including fishes, is caused by a failure to distinguish nociception from the psychological event that must be present if pain is to occur (Vierck 2006; Rose and Woodbury 2008). Of the fish species studied, brain structures that mediate pain and/or consciousness in mammals is lacking, and there is a deficiency in types of nociceptors (Rose et al. 2014).

The term "nociception" refers to the unconscious detection of potentially injurious stimuli by peripheral, spinal, and subcortical levels of the nervous system. Nociception is the neural process of encoding noxious stimuli, with responses that may be autonomic or behavioral (IASP 2011). While nociceptive responses often, but not always, precede pain in humans, they must be translated in specific regions of the conscious brain into a psychological experience in order to be classified and felt as pain. Nociception is a common, but not a universal, characteristic of vertebrates, however. For example, elasmobranch species studied appear to lack nociceptive capabilities (Coggeshall et al. 1978; Snow et al. 1993).

Since publication of the 2004 Guidelines (Use of Fishes in Research Committee 2004), a number of studies, principally by Sneddon and coworkers, have suggested that the pain experience was demonstrated by Rainbow Trout *Oncorhynchus mykiss* (e.g., Sneddon 2003; Sneddon et al. 2003a, 2003b; Reilly et al. 2008; summarized by Braithwaite 2010). The principal bases for their conclusions were observations of changes in fish feeding and ventilation rates, as well as "anomalous behaviors" subsequent to anesthesia and injections of large amounts of dilute acetic acid or bee venom into jaws of the trout. These experimentally induced behaviors have been challenged (by Rose 2003, 2007, and Rose et al. 2014) and have not been replicated by other investigators (Harms et al. 2005; Newby and Stevens 2008) or with other fish species tested (Reilly et al. 2008). Studies relying on endpoints of avoidance or escape from noxious stimuli as indicators of pain (Dunlop et al. 2006; Millsopp and Laming 2008) may also be inaccurate in that these behavioral endpoints do not require consciousness (see Rose 2007 and Rose et al. 2014).

Several studies of nociception in bony fishes have suggested some differences between teleost fishes and mammals that have bearing on the perception of pain. Anatomical and physiological studies have reported the occurrence of A-delta and C fiber nociceptor afferents in the trigeminal nerve of Rainbow Trout (Sneddon et al. 2003b) and Nile Tilapia *Oreochromis niloticus* tail nerves (Roques et al. 2010). A-delta fibers are the type of nociceptive afferent responsible for triggering rapidly sensed, well-localized "first pain" in humans, whereas C fibers are the most abundant type of mammalian somatosensory nerve fiber and are responsible for the more aversive, sustained, and burning type of "second pain" experienced by humans. Although these studies revealed that A-delta type fibers were fairly numerous, only a small number of C fibers were found in the trigeminal nerve of Rainbow Trout (Sneddon et al. 2003b) and the tail nerves of Nile Tilapia (Roques et al. 2010). Anatomic observations relative to these two species led Braithwaite (2010) to claim support for the plausibility of pain in teleosts, yet the rare occurrence of C fibers contraindicates the feasibility of pain-induced suffering, or even intense, prolonged nociception in fish. With elasmobranchs, in particular sharks and rays, neither A-delta nor C fibers have been found (Coggeshall et al. 1978; Leonard 1985; Snow et al. 1993). Elasmobranchs also appear to lack the spinal cord lamina I, a principal zone for synapsis of nociceptive afferent connections (Cameron et al. 1990).

Other experimental and field descriptive studies have contraindicated the pain experience in some fishes. Resumption of feeding and apparent normal activity have occurred immediately, or within minutes of recovery from anesthesia following surgery (Narnaware et al. 2000; Narnaware and Peter 2001; Harms et al. 2005). Biotelemetry studies have documented rapid recovery of normal behavior following transmitter implantation, as well as long-term survival and normal behavior (Wagner and Stevens 2000; Newby et al. 2007; Cooke et al. 2011). Studies of catch-and-release angling have consistently demonstrated the resumption of normal activity immediately after release, or at most within relatively short times of release. Many instances of fish being recaught within minutes of release have been reported (Schill et al. 1986; Rose et al. 2014). There is little debate that exposure to noxious stimuli, regardless of it being experienced as pain or not, is stressful from a behavioral standpoint; therefore, exposures to noxious stimuli should be minimized. While firm in believing that research on live fishes is acceptable and essential, the UFR Committee recognizes the sometimes difficult task facing IACUCs that must develop institutional guidelines that are both functional for, and accepted by, their constituents.

All subjects of experimental procedures must be protected from potential physiological or behavioral disturbances and harm in order for the results to be accepted as representative of the population from which the experimental subjects were drawn. Controlling and minimizing physiological stress and exposure to noxious stimuli reduces the possibility of harming experimental animals and improves data quality by minimizing any confounding effects of stress and other physiological/behavioral deviations, such as nociceptive behavioral responses that some observers might interpret as pain. The factors that are detrimental to fish welfare have been well delineated by valid, objective indicators of physiological and behavioral well-being. Scientific literature should inform an IACUC in developing specific policies, recommendations, or regulations concerning aquatic animal welfare.

Regardless of the conclusions accepted by individual investigators and their IACUCs concerning "pain," the importance of careful handling procedures, including sedation or anesthesia, is emphasized. The use of sedatives or anesthetics to restrain fishes is often essential to prevent harm to the animals, particularly where invasive procedures are involved (see section 7.12 Surgical Procedures). Sedation or anesthesia may also be important from the perspective of investigator safety, especially when handling large or otherwise hazardous subjects. Procedures described in the Guidelines provide additional information (see section 7.11 Restraint of Fishes: Sedatives and Related Chemicals).

5. Field Activities

5.1 Habitat and Population Considerations

Whether fishes are being collected live for investigations, preserved for study in a museum, or processed to obtain data needed for fisheries management, investigators should observe and pass on to students and employees a strict ethic of habitat conservation, and respectful and humane treatment of the animals in sampling, handling, and euthanasia (ASIH et al. 1987, 1988; AVMA 2013). Collecting should be conducted in a way that minimizes habitat disturbance and "excessive" mortality. The UFR Committee recognizes that currently no field collection techniques exist that will cause zero mortality events in the population being sampled. Research goals will generally dictate appropriate sampling methods. Given a set of alternative sampling methods and collecting gears, investigators can select the ones which cause the minimum levels of habitat disturbance and mortality in target and non-target fish populations. Gathering large series of animals from breeding aggregations should be avoided unless required to meet study objectives. Use of collecting techniques that damage habitat unnecessarily should also be avoided or performed to the minimum extent necessary to achieve study or sampling objectives. For example, trawling or other forms of dragged or towed gears is essential for documenting fish diversity or monitoring the health of fish populations; however, such gears can cause extensive disturbance to substrates, macrophytes, or other important structural elements of fish habitat. Sampling equipment and strategies can be designed to minimize incidental capture of non-target species. Collecting gears, such as gill nets deployed for nonlethal sampling, should be checked frequently to avoid unnecessary mortality. Regardless of the purpose of the experiment—whether to manipulate abundance or to study behavior, reproductive potential, or survivability—mortalities within the population and disturbance to habitat should be kept to the minimum amount that the investigator (along with the IACUC) determines to be acceptable.

The reader should note that some content in section 5 is not restricted to field activities but can extend to laboratory situations as well.

5.2 Field Collections

5.2.1 Permits

Research with fishes frequently requires capturing wild specimens from the field, whether for field-based studies—such as data recording, marking, and relocation—or for laboratory studies of live or preserved specimens. Except when collecting in the open ocean (waters not under the jurisdiction of any particular country), the collection of fishes for all research purposes requires a scientific collector's permit. Permits are issued by natural resource agencies of state, provincial, federal, and tribal entities in the United States and Canada (see section 3.4 Permits and Certificates). Permit applications generally request information about the research to be conducted, sampling methods, the areas to be sampled, and number and disposition of fish specimens to be collected. For a listing of state permitting agencies in the United States, as well

as other useful information about collection of fishes, see Walsh and Meador (1998). Collection of fishes on federal lands often requires a separate special use permit obtainable from the agency responsible for managing the land. The local, state, federal, and tribal authorities that issue collecting permits generally require collectors to notify them of the specific locations, dates, and proposed methods of sampling. Collection of fishes by federal personnel on private lands requires a permit approving access from the landowner.

5.2.2 Natural History Collections

Systematists and taxonomists interested in conducting studies on preserved fishes should be aware of the wealth of specimens archived in natural history collections (see section 5.2.5 Museum Specimens and Other Preserved Specimens) before considering the removal of additional animals from the field. Repeated collections, however, are often warranted to provide information on temporal changes in the study population. The holdings of many ichthyological collections are accessible through database network portals such as Fishnet2 (http://fishnet2.net/) and the Global Biodiversity Information Facility (http://www.gbif.org/). Many state agencies and universities have accessible natural history collections. For listings of fish collections in the United States and Canada, see Leviton et al. (1985), Poss and Collette (1995), and Walsh and Meador (1998). A listing of institutional resource collections available internationally in herpetology and ichthyology, along with symbolic codes and citations, is made possible through ASIH (Sabaj Perez 2013, http://www.asih.org/resources/standard-symbolic-codes-institutional-resource-collections-herpetology-ichthyology).

5.2.3 Representative Samples

Generally, the questions being explored and the study design itself dictate the number of specimens required for an investigation. Acquiring fishes for study generally involves the taking of a very small portion of the population or community present at a location. The general principle applied when sampling fishes is to take the fewest animals necessary to reliably address the hypothesis (see section 2.3 Statistical Design). The minimum number of fishes necessary to provide robust statistical results should inform the sampling protocol. Depending on the gear and methods, and the amount of handling required, high mortality rates may result. This is especially true in investigations involving fish eggs and early life stages. However, high levels of juvenile mortalities and rapid recoveries from population reductions are both characteristic events in the life histories of many fish species.

Sampling by using visual surveys alone is not always sufficient. This is the case in habitats that are structurally and biologically complex, where fish biodiversity data is necessary for their conservation and management. Small, cryptic fishes in coral-reef habitats, for example, are best collected by using small-scale sampling with ichthyocides; increased collection percentages of visually detected fish occurred with ichthyocide application (Smith-Vaniz et al. 2006; Ackerman and Bellwod (2000). The most commonly used ichthyocide is rotenone (see section 8.1

Euthanasia), a naturally occurring ketone from leguminous plants native to Southeast Asia and South America. The use of this chemical option has been diverse (McClay 2000); its use had been indicated with the threat of exotics (Rayner and Creese 2006). Robertson and Smith-Vaniz (2008) reviewed rotenone used by indigenous subsistence fishers and by fishery managers, as well as its toxicity and effects on other organisms. Rotenone anesthetizes and dispatches fishes by blocking the cellular uptake of oxygen (Singer and Ramsay 1994). A manual and SOP (http://fisheries.org/shop/55061p.) detail the proper use of rotenone (Finlayson et al. 2000; Finlayson et al. 2010), and numerous training courses are offered for fishery biologists and public agencies. Finlayson et al. (2010) recommended cautionary use of rotenone as a last resort due to potential harm to unintended targets. Investigation into alternative methods (Marking 2011) is prudent, as is the availability of taxonomic expertise (Walsh and Meador 1998) so to confirm the species present (see section 8.1 Euthanasia).

Sampling fish in contaminants studies is often inherent in biomonitoring of aquatic ecosystems because of capabilities of fish to accumulate environmental contaminants and to respond physiologically. Field procedures for sampling fish for chemical contaminants (Hughes et al. 2006; Schmitt et al. 1999) are useful, with protocols chosen according to study endpoints. A suite of documents and databases are available from USGS Biomonitoring of Environmental Status and Trends (BEST) program (http://pubs.er.usgs.gov/publication/itr19990007) and National Contaminant Biomonitoring Program (http://www.cerc.usgs.gov/data/ncbp/ncbp.html.

5.2.4 Collection of Imperiled Species

The term "imperiled species" applies not only to species officially listed as threatened or endangered by state or federal agencies but also to species that have been identified as candidates for such listings. The number of endangered, threatened, and vulnerable fish species in the southern United States has increased 125% between 1969 and 1989 (Warren et al. 2000). Investigators need to be aware of whether an aquatic habitat to be sampled supports imperiled species, as well as how to identify those species in the field (Warren and Burr 1994). Investigators can also determine if the habitats that support imperiled and nonimperiled species are considered areas of conservation concern and if species could be a focus of conservation concern (Jenkins et al. 2011). State wildlife action plans (Association of Fish and Wildlife Agencies 2007, http://www.teaming.com/state-wildlife-action-plans-swaps) and the network of U.S. Natural Heritage Programs (http://www.natureserve.org/natureserve-network) maintain listings of fishes (and other animal and plant species) of conservation concern. 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, http://www.natureserve.org/visitLocal/index.jsp) and the USFWS. The USFWS Endangered Species Program (http://www.fws.gov/endangered/) maintains lists of federally protected species. The list of federally threatened and endangered fishes may be also be searched on Web sites of the National Oceanic and Atmospheric Administration (NOAA, http://www.noaa.gov/)

Fisheries Service (http://www.nmfs.noaa.gov/) Office of Protected Resources (http://www.nmfs.noaa.gov/pr/) Web site or NatureServe (http://www.natureserve.org/). A bulletin highlighting protected marine or anadromous fishes (http://www.nmfs.noaa.gov/pr/species/fish/) is also available from NOAA Fisheries Service. Lists of protected fishes in Canada, Mexico, and other foreign countries can be viewed online on the respective national Web sites (such as the Species at Risk Act Public Registry, http://www.sararegistry.gc.ca/default_e.cfm of the Canadian Wildlife Service), as well as via the "Foreign Species" report on the USFWS Endangered Species Program Web site (http://ecos.fws.gov/tess_public/SpeciesReport.do?lead=10&listingType=L). The International Union for Conservation of Nature and Natural Resources (IUCN) maintains The IUCN Red List of Threatened Species™, version 2013.1 (http://www.iucnredlist.org/), providing global coverage of the conservation status of freshwater and marine fishes, as well as other plants and animals.

The collection of imperiled species is allowed only under special circumstances (e.g., conservation status surveys) and requires special permits. Only noninvasive handling techniques (handling that results in no harm whatsoever to the animal) are to be used. Examples can include blood and milt collection, and certain fin clipping and tagging methods. If the goal of the research is to collect an imperiled species for live study, or if incidental capture is anticipated as bycatch, then any collection methods that may be injurious (e.g., gill net catch without close monitoring) or lethal (e.g., ichthyocides) should be avoided.

Conservation efforts for imperiled fish species frequently involve translocations, either among natural localities or from nature to propagation facilities and 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 the appropriateness of transplanted individuals among sites (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 unauthorized individuals.

5.2.5 Museum Specimens and Other Preserved Specimens

The collection of fishes from natural populations for museum preservation is critical for (1) understanding basic biology and life history, (2) documenting and recording biodiversity, and (3) establishing reference collections essential for understanding evolutionary relationships and environmental effects (ASIH et al. 1987, 1988). Studies of ecosystem variation or delineation of new species frequently require collection of relatively large series (sufficient for computing statistics on counts and measurements) from multiple populations across geographic ranges (Hughes and McCormick 2006). Sampling natural fish populations for these purposes typically involves broad surveys and collection of specimens in proportion to their occurrence in natural

populations; moreover, such sampling may not be hypothesis-driven. Studies of molecular systematics typically involve very small numbers of specimens, or small amounts of tissue removed from study fishes. However, it is just as important in these studies as in general ecological surveys to deposit voucher specimens in natural history museums, where samples are maintained frozen or preserved in a fixative such as 95% alcohol (isopropanol) or 70% ethanol, for future reference (Wheeler 2003). Museum collections of fishes are also available for use in other types of research. Two important principles that should be followed in collecting fishes for museum preservation are (1) the numbers of specimens collected should be the minimum necessary to accomplish study goals, and (2) animals collected should serve a variety of studies. Precise notations containing specific field data (such as date, exact location, habitat type, etc.) should accompany each collection.

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 (3.7% formaldehyde) solution, followed by transfer to alcohol (70% ethanol, un-denatured preferred) for long-term preservation and storage, as with voucher specimens. Chemicals are often 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) (Luna 1992, 1992; Presnell et al. 1997; Clark 1981). Fixation by these methods typically involves small pieces of tissue dissected from specimens that may be sacrificed by means other than immersion in formalin. Carcasses for long-term archiving as voucher specimens should be fixed in formalin and later transferred to alcohol. Euthanizing fish prior to immersion in formalin should be practiced, provided that the sedative does not cause effects detrimental to the objectives of the research. A variety of chemicals, such as tricaine methanesulfonate (MS-222), may be used to anesthetize or euthanize fishes (see section 7.11 Restraint of Fishes: Sedatives and Related Chemicals). When study interests demand that specimens be fixed without prior treatment with sedatives, the specimens can be numbed in ice water, or for small fishes, immersed directly in liquid nitrogen (see section 8.1 Euthanasia).

Portions of animal specimens, including sperm, ova, embryos, tissues, and serum, are sometimes tissue banked. For example, the National Animal Germplasm Program (http://nrrc.ars.usda.gov/A-GRIN/main_webpage/ars?record_source=US) acquires and preserves genetic resources to secure biological diversity for population reconstitution or genomic studies. The San Diego Zoo Institute for Conservation Research (http://www.sandiegozooglobal.org/ICR/purpose) uses stored genetic resources in multiple technologies. Various iterations of specimen banking for retrospective analyses occur globally

for a multitude of investigations, including environmental monitoring, genetics research, and systematics. Fish tissue (liver and muscle) has been collected for the long-term storage of a variety of environmental specimens by the National Institute of Standards and Technology (NIST, http://www.nist.gov/index.html) through the National Biomonitoring Specimen Bank (Wise and Koster 1995; Becker and Wise 2006).

5.3 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 and stress (see section 4.2 Stress) (McMichael et al. 1998; Henry and Grizzle 2003; Henry et al. 2003). Live wells or tanks should be provided if fishes are to be kept for more than the time needed to collect essential metrics. Care should be taken to avoid accidental capture of nontarget species and to ensure release of incidentally collected individuals with minimal or no 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 sections 5.5 Dangerous Species and Specimens and 7.10 Dangerous Species and Specimens in Captivity).

Several studies have shown electrofishing to be among the most effective techniques for obtaining fish assemblage data in freshwater habitats (Yoder and Smith 1998). 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 (e.g., conductivity, water depth, and presence of obstructions), as well as use of gear and techniques that minimize potential for electrofishing injury to fishes (Snyder 2003; Dean and Temple 2011). Alternative sampling methods, such as seining, gill or trammel nets, trawls, cast nets, lift or push nets, rigid traps (e.g., minnow traps, slat traps), hoop nets, fyke nets, weirs, or angling, can be just as injurious to fishes if not conducted properly. The sampling methods chosen should allow for efficient capture of the species and sizes of fish needed to address research objectives while minimizing injury and mortality of collected fishes and non-target organisms. Multiple sampling gears may be required for the collection of a broad range of fish sizes or species or if diverse habitats are covered. Passive capture methods, such as set nets and traps, should be checked frequently enough to prevent unnecessary mortality of both target and non-target species. Nets and traps should be carefully positioned, anchored, and flagged and then removed at the cessation of sampling to avoid “ghost fishing” (lost or abandoned fishing gear that continues to kill fish and other sea life). Bonar et al. (2009) and Zale et al. (2013) provide additional information concerning standard sampling methods for fishes in freshwater environments, as well as the efficiency and specificity of various collecting gears.

5.4 Field Restraint of Fishes: Sedatives

Prolonged restraint that causes physiological stress should be avoided. In some cases, use of a sedative or anesthetic agent to minimize stress may be advisable. Although the terms

"anesthesia," "sedation," and "immobilization" have been used interchangeably in referring to fishes, the words have distinct definitions reflecting different levels of sensory perception and responsiveness. Ross and Ross (2008) have defined anesthesia as "a reversible, generalized loss of sensory perception accompanied by a sleep-like state induced by drugs, or by physical means" and sedation as "a preliminary level of anesthesia, in which response to stimulation is greatly reduced and some analgesia is achieved, but sensory abilities are generally intact and loss of equilibrium does not occur." "Immobilization" generally refers to prevention of movement and does not imply any status regarding the acuity of sensory perception. Depending on the chemical agent and its mode of action in fish, one or more of these terms may apply. As a conservative approach, the term "sedative" is used herein, as this term may be applicable for most of the agents used in restraining and in facilitating the handling of fishes.

5.4.1 Drugs Approved for Use on Fish

The AFS, the AADAP Program, and the FDA Center for Veterinary Medicine engage in ongoing dialogs to ensure fisheries professionals have access to the most current and accurate information regarding the use of fish drugs (Bowker and Trushenski 2013). The only substance approved by the FDA for field sedation of fishes is MS-222. The product is currently available in the United States and contains Tricaine-S™ (Western Chemical, Inc.; Ferndale, Washington). However, use of MS-222 in the field is limited because of an FDA requirement that food fish, including feral fishes that may be caught and eaten by humans, must go through a 21-day withdrawal period prior to release or slaughter for human consumption (Anderson et al. 1997; Trushenski et al. 2012a, 2012b). Use of MS-222 is further restricted to ictalurids, salmonids, esocids, and percids or other laboratory or hatchery fishes held at water temperatures greater than 10°C. When handling the dry form of the chemical, personal protective equipment such as a respiratory mask and gloves should be used. See Coyle et al. 2004 on anesthetic use with aquatic animals in Southern Regional Aquaculture Center fact sheet 3900 at https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/162/. Unlike many therapeutic drugs, these sedatives cannot be prescribed for extra-label uses (i.e., for other taxa, or treatment regimens not written on the label). Addition of an appropriate buffering compound, such as sodium carbonate, to MS-222 is recommended when used in "soft" water to counteract its acidifying effects, thereby increasing post-treatment survival (Smit et al. 1979).

5.4.2 Low Regulatory Priority (LRP) Drugs

Although carbon dioxide (CO_2) is not FDA-approved as a drug for use on fish, it is considered a drug of LRP and can be used as a fish sedative provided certain conditions are met (FDA 2011; see Appendix Table 1 for a list of LRP drugs and considerations for use). More specifically, if an appropriate grade of CO_2 is used, good management practices are followed, and local environmental requirements are met, the FDA has determined that regulatory action against the use of CO_2 as a fish sedative is unlikely.

5.4.3 Investigational New Animal Drugs (INAD)

Two currently unapproved drugs, a benzocaine-based product (Benzoak is 20% benzocaine; sponsored by ACD Pharmaceuticals AS, Ålesund, Norway) and a eugenol-based product (AQUI-S20E is 10% eugenol; sponsored by AQUIS New Zealand Ltd., Lower Hutt, New Zealand) may have initial FDA approval for use to sedate finfish in 2015 and may be used as sedatives under authorization of an INAD exemption granted by the FDA and held by the USFWS. Currently, the eugenol-based product can be used as an immediate-release sedative for field applications where it is likely that fish will be sedated just once in their lifetime. All other applications require a 3-day withdrawal period. The AADAP Research Program (http://www.fws.gov/fisheries/aadap/research%20program%20-%20history.htm) is responsible for the USFWS INAD Program (http://www.fws.gov/fisheries/aadap/national.htm), encompassing regulatory and research studies for aquaculture drug use. Updates on aquaculture drugs are found through the INAD program Web site.

With the use of any sedative, a small number of fish should be tested to determine a suitable dose within the allowable ranges and to ensure that the species will return to normal physiological and behavioral status within an acceptable recovery time. The animals must be kept under observation until appropriate recovery occurs (see section 4.2.2 Measuring and Avoiding Stress). Used sedatives must undergo disposal in accordance with local, state, tribal, provincial, and federal regulations (see also 5.7 Facilities for Temporary Holding and Maintenance and 7.11 Restraint of Fishes: Sedatives and Related Chemicals).

5.5 Dangerous Species and Specimens

Species considered dangerous to humans are most often encountered under field conditions, yet the guidelines are similar for laboratory situations. Dangerous species should be handled in a manner that is safe for both the investigator and the animal being handled. Investigators should be cognizant of safety regulations for their institution regarding the use of dangerous or venomous animals. Those regulations may include SOPs that limit access for only authorized personnel, 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. General guidelines follow:

- Procedures should minimize handling time required and reduce or eliminate contact between the handler and the animal.
- The investigator should not work alone. A second person, also knowledgeable in capture and handling techniques, and emergency measures, should be present at all times.
- To prevent serious secondary infections, the investigator should take care to avoid the commonly occurring bites, scrapes and abrasions, cuts, and spine punctures, all of which can lead to infections.

- Human wounds resulting from improper handling of marine and freshwater animals should be cleansed as soon as possible to avoid bacterial infection (i.e., zoonosis). Any bleeding needs to be controlled.
- Wounds should be irrigated with at least a liter of the cleanest disinfected fresh water available. In the absence of sterile saline or sterile water, tap water or bottled drinking water can be used, whereas ocean water is to be avoided.
- After routine first aid has been applied, medical assistance should be sought to prevent complications.

Overall, consulting the relevant literature and colleagues experienced with the species is of primary importance. For general as well as specific information, with special reference to the marine environment, several books are available that are primarily written as cautionary first aid guides for scuba divers, free divers, and snorkelers who frequently come into contact with marine animals (Halstead 1995; Cunningham and Goetz 1996; Auerback 1997), as is information on the Divers Alert Network (DAN) Web site (www.diversalertnetwork.org).

5.6 Handling and Transport

Fishes 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 water quality changes, 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 (see section 4.2 Stress).

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 fishes 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 1993b). Depending on the severity of the stressors and exposure time, mortality can result from osmoregulatory dysfunction and immunosuppression.

To mitigate stress associated with handling and transport, the investigator can reduce the number and severity of the stressors, minimize the duration of stressors, and minimize increases in

metabolic rate. Harvesting techniques and preshipment treatment are important to the successful shipping of live fish (Dupree and Huner 1984). Preconditioning treatments can involve the addition of sedatives 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 should 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 ranges of dissolved oxygen, carbon dioxide, temperature, ammonia, and pH during transport is essential. Fishes 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 fishes in a container of water and minimizes direct contact with nets. Wet transfer usually results in less stress than dry transfer, where the net is used alone. Ideally, fishes 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 vary depending upon conditions, the amount of handling, and research objectives, but 72 hours typically is considered a minimum following extensive handling (see section 5.8 Field Acclimation).

5.7 Facilities for Temporary Holding and Maintenance

Because the biological needs of each aquatic species and the nature of individual projects vary, only the most general recommendations are provided on temporary holding and maintenance. Testing and comparing several methods of housing may be necessary in order to find the most appropriate for the needs of the species and the purpose(s) of the study. 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 animal 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 factors is under investigation for achieving a desired effect (e.g., spawning cycle manipulation) (ASIH et al. 1987, 1988). Fish species have optimal thermal regimes (Sylvester 1970), and the immune system functions best within such ranges (Bly and Clem 1992). Diseases occur during temperature windows as well, such as *Edwardsiella ictaluri* in Channel Catfish (Hawke 1979). 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 sportfish species, fishes are generally held in vats or tanks before shipment. This holding enables the producer to grade fish according to size and to administer drug therapies if necessary. Holding also acclimates the fish for handling and transport (Huner et al. 1984). When Channel Catfish are harvested from a pond, live cars or fish holding bags are used in the industry (Huner et al. 1984; Green and Yant 2011) 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, fishes might be moved to deeper water in which cases the use of recirculating pumps or aerators can be beneficial.

As with other containment systems, the holding tank needs to allow for the stocking density or the relation of fish biomass to available water volume. Water inflow and turnover rate must be considered because sufficient water exchanges are needed for good water quality. Oxygen available in the incoming water needs to exceed the metabolic oxygen consumption by fishes in the tank (Casebolt et al. 1998). Sufficient aeration can be supplied by compressed air, injected or bottled oxygen, or agitation. Sedatives can also be used to reduce the physical activities of fishes, if consistent with research objectives. Excess noise and vibrations should be avoided because such factors can produce acute or chronic stress response in fish (Stoskopf 1992) (see section 7.4 Facilities for Long-Term Housing of Fishes).

If extreme weather and environmental events occur, emergency preparedness measures may be necessary for future short-term maintenance of research animals. For instance, excess feed storage, alternative water supplies, and back-up generators may need to be in place. Proactive institutional and researcher plans may be practical and even required by IACUCs.

5.8 Field Acclimation

Because numerous physiological processes can be altered upon handling and transferring fishes, acclimating or conditioning fish to their new environment lessens potential negative effects. If the physical and chemical qualities of the water supply for the temporary holding facility (see section 5.7 Facilities for Temporary Holding and Maintenance) are different from those of the source water, care should be taken to provide water as similar as possible. For example, fish in floating plastic bags with an atmosphere of oxygen above the water may be used to allow the captured fish to acclimate to the new water temperature. If differences are more substantial, gradually replacing the water in transport units with source water from the holding unit is a common practice that provides adequate time for fish acclimation. Useful notes on how to transport and acclimate live warmwater fishes are summarized in the Southern Regional Aquaculture Center Transportation of Warmwater Fish factsheets Loading Rates and Tips by Species (Jensen 1990a, https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/77/),

Equipment and Guidelines (Wynne and Wurts 2011, https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/74/), and Procedures and Loading Rates (Jensen 1990b, https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/76/), as well as in the North Central Regional Aquaculture Center factsheet, Transportation of Fish in Bags (Swann 1993, http://www.ncrac.org/oldfiles/NR/rdonlyres/237DFD95-2967-4455-A668-3CFA051036BE/0/ncrac104.pdf).

5.9 Collection of Blood and Other Tissues

Results obtained from careful collection and examination of blood and other tissues are often critically important to research on fishes (Blaxhall 1972; Fange 1992). Sterile conditions for these procedures are often impossible to provide under field conditions, and care must be exercised to prevent injuries and stresses to the animals. Samples of blood and body fluids can be obtained from fishes without compromising their survival, even from small specimens under 100 grams (Stoskopf 1993a). Plastic syringes containing a small amount of anticoagulant such as sodium- or ammonium heparin or sodium citrate are suggested to prevent blood clotting. Study objectives will determine the proper selection of type, volume, and concentration of anticoagulant, if needed. Three main techniques have been devised for collecting blood from fishes: cardiac puncture, venous puncture, and caudal bleeding (Blaxhall 1972; Stoskopf 1993a, b). 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 may be 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 sedated fish that will be euthanized following the procedure. However, extraneous fluids and proteins that may influence cell quality often co-occur with this procedure. Caution must be exercised to ensure that the method of sedation will not interfere with subsequent analyses. Additional information on sampling methods for the collection of blood from fishes 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 typically requires sacrifice of the subject animals and must be preceded by appropriate anesthesia or euthanasia (see section 8.1 Euthanasia). These tissues can also be used for such purposes as contaminants analyses (see section 5.2.3 Representative Samples), or for biopsy or necropsy. Tissues may be used fresh or frozen, or placed in a fixation or preserving medium such as buffered formalin, ethanol, or methanol and then histologically processed (Luna 1992; Presnell et al. 1997). 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 live fish and may be considered non-

invasive sampling. This is important when working with imperiled species and small populations (see section 5.2.4 Collection of Imperiled Species).

When transporting live tissues, the medium must have appropriate ionic and osmotic concentrations and may contain a sugar as an energy source. Experienced investigators have found Hank's Balanced Salt Solution (Jenkins et al. 2013), 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 (Jenkins 2011a; Jenkins et al. 2013). Certain cell and nucleic acid stabilizers can make sampling of fish possible from remote locations for later tissue analysis in the laboratory (Olivier and Jenkins, in press).

6. Marking and Tagging

6.1 General Principles

Tagged and marked animals have been studied to obtain information on their behavior, population dynamics, and ecology, all of which are essential for developing conservation and management strategies. Identification of fishes by using naturally occurring or artificial tags or marks is often required for studies on age and growth, mortality rates (including natural and fishing-induced mortality), abundance, angler catch or harvest rates, habitat use and movement/migration, stock recognition, or stocking success (Pine et al., 2012). Investigators can use both intrinsic and extrinsic identification systems, allowing the nature of the study to dictate the type of tag or mark employed. Integrated use of more than one tagging or marking technique helps ensure fish identification and is helpful in estimating tag loss rates.

Basic considerations for selecting a particular type of tag or mark in the context of the study objectives include potential effects on animal survival, behavior, and growth; tag permanency and recognition; number and size of the animals; stress of capture, handling, and marking; total costs; recovery of the marked fishes; and any required coordination among agencies, states, provinces, or countries (Pine et al. 2012). 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, as well as other potential impacts (ASIH et al. 1987, 1988). Because techniques for tagging and marking fishes have been extensively reviewed and are constantly evolving, literature reviews should inform the researcher (McFarlane et al. 1990; Parker et al. 1990; Nielsen 1992; Hammerschlag et al. 2011; Wagner et al. 2011; McKenzie et al. 2012; Pine et al. 2012).

The effects of marking on fishes depend on the physical condition of the fish at the time of release. Occurrence of injury is species and size specific, and smaller fishes may be more susceptible. Minor wounds caused by most tagging and marking procedures typically heal satisfactorily without treatment with antibiotics. All sedatives or antibiotics administered must be used in a manner consistent with regulatory requirements.

6.2 External Tags and Marks

The use of external tags and marks has evolved over a long period of time (McFarlane et al. 1990). Both natural marks and artificial tags or marks are in common use in fisheries research, and each type offers different capabilities, as well as limitations. Natural, external marks include meristic characteristics, pigmentation, morphometric measures, and scale characteristics, but natural marks are subject to environmental and genetic influences. Fish scale shape and size, as

well as circulus spacing, are frequently used. The effective use of natural marks requires being well informed on fish life history.

Multiple methods are available for generating artificial external marks on fishes. Alteration of fins or other body parts, in practice for over 100 years, can be accomplished by clipping or hole punching. The selection of fins for clipping or removal is dependent upon the species under study; for example, clipping the anal fin of poeciliid males would be inappropriate because it functions as a copulatory organ, yet removal of the adipose fin of a salmonid would have negligible impacts (ASIH et al. 1987, 1988) (see section 5.2.4 Collection of Imperiled Species). Hot or cold branding, the process of marking by placing an apparatus (e.g., liquid nitrogen brand) against the body for a few seconds, may be an effective marking technique in specific situations and does not cause substantial injury to underlying fish tissues (Bryant et al. 1990). Fishes should be anesthetized prior to branding. External colorants for marking fishes include dyes, stains, inks, paints, liquid latex, visible implant elastomers, and plastics that are administered by immersion, spraying, injection, or tattooing. Care is needed for distinguishing external colorant marks of similar tones (Curtis 2006).

External tags are conspicuous by their color, shape, size, or location of attachment and are composed of various materials. The print on external tags can relay important data, such as individual fish identification codes, reward value for capture and tag return, and the investigator's contact information. Designed for hydroturbine passage survival studies, an external transmitter that is molded to the fish has shown utility (Deng et al. 2012; Brown et al. 2013). External tags commonly applied to fishes include dart and t-bar anchor tags, disc tags, Carlin tags, and spaghetti or loop tags (Guy et al. 1996). Dart and anchor tags are the most frequently used external tags (Nielsen 1992), but a high loss rate has been reported in some species (Guy et al. 1996). Proper insertion technique and use of small tags relative to fish size can reduce the potential for fish injury and tag loss (Guy et al. 1996).

6.3 Internal Tags and Marks, and Biotelemetry

Implanted coded wire tags, radio and acoustic telemetry transmitters, archival biologgers, passive integrated transponder (PIT) tags, visible implanted alpha numeric tags, otolith marks, and natural parasites are internal marking systems used to identify fish (Prentice et al. 1990; Brown et al. 2011). The use of a coded wire tag identification system has been tested for management and research applications with multiple genera of fishes (Buckley and Blankenship 1990) including juvenile salmonids (Liedtke et al. 2012), and adverse tissue reactions did not occur. The coded wire tag is normally injected into cartilage, connective tissue, or muscle and is detected electronically later with a handheld device.

A PIT tag consists of a small computer chip and antenna enclosed in a glass tube that is injected into the fish's musculature or peritoneal cavity. Each PIT tag carries a unique code that is

relayed to a handheld or stationary reading device when the tag is within range. Advantages of PIT tags include a long lifespan and generally a high retention rate (Freeland 1995; Guy et al. 1996); however, tagging location within the body and fish length can influence retention rate (Guy et al. 1996; Rude et al. 2011). The PIT tag data can be read through soft and hard fish tissue; in seawater and freshwater; through glass, plastic, and metal containers; and when fishes are moving at some velocity. Above certain fish size thresholds (Tatara 2009), they have little or no effect on fish growth, survival, or behavior (Prentice et al. 1990). Various tags and methods are available for the PIT tagging procedures; information can be found at state websites (e.g., Idaho and Arizona) and at manufacturer websites.

Visible implanted tags are alphanumerically coded and made of polyester film. They are inserted subcutaneously into transparent tissue so that they remain externally visible (Haw et al. 1990). Common tagging locations include transparent tissues posterior to the eye, in the lower jaw, or in fin membranes. Tag retention varies by species, tag location within the body, and fish size; very small fishes may have insufficient transparent tissue to accommodate the size of the tag (Griffiths 2002).

Manipulating environmental temperature, feeding rates, photoperiod, external chemical baths, or labeled feeds can induce specific marks in fish otoliths. Fishes being propagated under controlled conditions are ideal for such manipulations. Otolith microstructural features and induced marks are permanent and can be viewed and analyzed in fish of any age. Tetracycline and other fluorescent compounds (e.g., calcein, alizarin compounds) are well-known markers for calcified structures in fishes (Guy et al. 1996; Carty and Bowker 2013), although such applications are regulated by the FDA as drug treatments (see section 5.4.1 Drugs Approved for Use on Fish). Fish size, compound dosage and uptake method, and water chemistry can influence marking success with fluorescent compounds (Beckman et al. 1990; Rutherford et al. 2002). Marking success is highest during times when fish growth is rapid (Conover and Sheehan 1999). Otoliths and other calcified structures can also be marked with alkaline earth and rare earth elements (Behrens Yamada and Mulligan 1990) or isotopically labeled compounds (Munro et al. 2008; Smith and Whitledge 2011). Injection of gravid females with compounds enriched with a particular chemical element or stable isotope can be used to "mass mark" embryonic otoliths of offspring (Thorrold et al. 2006). Fisheries that require stock definitions and assessment of stocking success or dispersal of early life stages are well suited to otolith-marking techniques.

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 fishes (MacKenzie 1983). Recovery of internal parasites used as biological tags is enhanced if parasites are associated with a specific anatomical site on 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 in one fish population to its incidence in another (Wydoski and Emery 1983).

Underwater biotelemetry involves attaching a device that relays biological information via ultrasonic or radio signals from a fish to a remote receiving system (Cooke et al. 2012). Radio transmission is practical only in freshwater at relatively shallow depths (ASIH et al. 1987, 1988). The selection of a tag or transmitter and the method and site of attachment or implantation is to be appropriate for the species and size of fish and performed by trained personnel. Surgical implantation of transmitters into the coelom is common with free-ranging fishes. Use of the smallest and lightest transmitter that provides the desired signal type, strength, and battery lifespan will minimize tag loss and potential effects of transmitter attachment on fish survival, growth, and behavior. Wagner et al. (2011) and Mulcahy (2003, 2013) have reviewed surgical techniques for implanting transmitters in fishes. External, neutrally buoyant transmitters have been developed for turbine-passage studies with juvenile salmonids at hydroelectric facilities (Deng et al. 2012). With fish exposed to rapid pressure changes, external transmitters may decrease the likelihood of injury or death compared to surgically implanted transmitters (Brown et al. 2013). Techniques to minimize skin irritation should be used following attachment of external transmitters (Crook 2004) (see section 7.12 Surgical Procedures).

6.4 Genetic Markers

The development of techniques employing markers based on chromosome and nuclear DNA polymorphisms has been rapid and continues to evolve. Benefits have emerged for using DNA marks in selective breeding programs, in evaluating the contribution and effects of stocked species, and in delineating specific habitat requirements for hatchery-produced fish (Purdom 1993). For managing natural populations, knowing whether the fish species exists as a single genetic unit or relatively genetically distinct groups is critical (Beaumont and Hoare 2003). Genetic tagging has been effective even in a large population of wide-ranging and inaccessible mammals such as cetaceans (Palsbøll et al. 1997). "DNA barcoding" has been useful in species identification and in accurately labeling seafood products (Handy et al. 2011). An additional incentive for the use of genetic tagging is that adequate tissue samples can be obtained nonlethally (e.g., fin clipping) and with minimal handling. Genetic tags are permanent and exist in all individuals, thus representing a good alternative to traditional tags.

Prior to the development of DNA techniques for differentiating fish populations, investigators studied allozymes—variant enzyme forms that are coded by different alleles at the same locus or DNA sequence. This type of genetic analysis sometimes required sacrificing fish to obtain appropriate samples, and with karyotype analysis, the examination of dividing cells was required. Small laboratory fishes such as Japanese Medaka and Zebrafish were used extensively as models for studies in vertebrate developmental genetics and for transgenic investigations

(Ozato and Wakamatsu 1994). The use of DNA markers for fish stock identification was initially limited to differences in mitochondrial DNA (Phillips and Ihssen 1990).

Genetic variation at the DNA level can be measured in multiple ways (Beaumont and Hoare 2003). Some include DNA length and sequence variations, DNA fragment size variations with such techniques as restriction fragment length polymorphisms (RFLPs), RFLPs with mitochondrial DNA, variable number tandem repeats (VNTR) (microsatellites), DNA fingerprinting using restriction enzymes, random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). Next-generation sequencing technologies rapidly obtain short DNA sequences at thousands of loci, providing a depth of potential for gathering genomic information (Mardis 2008).

Further, genetic tags can be used to address questions on evolution, demographics, and behavior (Palsbøll et al. 1997), in addition to monitoring performance traits such as long-term reproductive success and effects of habitat restoration and conservation efforts. Fisheries scientists dealing with such questions will need to update their knowledge of the appropriate, scientifically accepted genetic identification systems for their potential applications (Lincoln 1994; Poompuang and Hallerman 1997).

6.5 Stable Isotopes

Stable isotopes are nonradioactive, naturally occurring forms of chemical elements that do not decay spontaneously and are generally energetically stable. Stable isotopes of a particular chemical element differ in mass but otherwise have equivalent chemical properties. In contrast to radioisotopes, which are tightly regulated, the use of stable isotopes does not require specially approved facilities and permits. Isotope fractionation has been studied for many years in natural systems, and stable isotope ratios are now used with relative frequency for fish marking. Stable isotopes can inform studies on trophic food-web structures, feed efficiencies, fish migration and places of origin, contaminant bioaccumulation, and other physiological and ecological processes. A variety of elements (e.g., H, C, N, O, S, Sr, and Ba) can be used as natural markers, or the elements can be artificially administered. Variation in the ratios of heavy to light stable isotopes of a particular element (expressed as δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$, or $^{87}Sr/^{86}Sr$) can be measured with a high degree of accuracy and precision and can be used to identify sources of these chemical elements and trace them within individual animals, populations, or ecosystems.

For obtaining fish tissues, sedation may be required (see section 7.11 Restraint of Fishes: Sedatives and Related Chemicals) or sacrificing may be necessary. Depending upon the objectives of the research, nonlethal sampling may be possible by using scales, sectioned fin rays or spines, fin clips, or muscle tissue samples obtained with a small biopsy punch for stable isotope analyses. Sampling of otoliths as metabolically inert structures is also common. Structures such as otoliths or fin rays or spines offer investigators a chronological record of

isotopic signatures and the opportunity to track movements and/or food sources through the lifespan of the organism (Chapman et al. 2013). Different types of metabolically active tissues have different elemental turnover rates; therefore, each investigator must determine which tissues may provide materials needed to satisfy the requirements of the studies. Representative information on the use of stable isotopes in animal ecology has been provided by Fry (2006) and Rubenstein and Hobson (2004). Elsdon et al. (2008) have reviewed otolith chemistry as a technique for determining environmental history of fishes.

6.6 Fatty Acids

In a manner similar to stable isotopes, fatty acids can be used as biomarkers to identify nutrient pathways in food webs, predator-prey relationships, and the relative contributions of allochthonous (remote) versus autothonous (local) inputs. The use of fatty acids as biomarkers is based on the principle that fishes and many other aquatic organisms are composed of what they have eaten. Once consumed, fatty acids may be catabolized for energy or biotransformed, so the fatty acid profiles within tissues tend to reflect the dietary fatty acid profile. Some fatty acids cannot be synthesized by vertebrates (i.e., 18:2n-6 [linoleic acid] and 18:3n-3[alpha-linolenic acid]) or are not synthesized in appreciable amounts relative to dietary intake (e.g., 20:4n-6 [arachidonic acid], 20:5n-3 [eicosapentaenoic acid], and 22:6n-3 [docosahexaenoic acid]); thus, they are particularly useful indicators of recent nutritional history. As mentioned for stable isotopes, various tissues have different metabolic turnover rates; thus, to be accurate, efforts linking tissue fatty acid profiles with chronological records of feeding behavior involve validation studies to account for establishing rates of profile change. Different lipids classes and tissue types have "signature" profiles making them variably responsive to such changes as dietary intake and environmental conditions. For example, phospholipid profiles tend to include certain saturated fatty acids (e.g., 16:0) and polyunsaturated fatty acids (e.g., 18:2n-6 and long-chain polyunsaturated fatty acids), whereas triacylglyceride profiles tend to have a more diverse fatty acid composition. Traditionally, muscle and liver tissues have been used for analyses, and sacrificing the animal has been necessary. However, adipose fin clips have shown utility for such analyses (M. Young, J. Trushenski, and G. Whitledge, unpublished data). For more information on the role of fatty acids in aquatic ecosystems and the use of fatty acids as biomarkers, see Arts et al. (2009); for more information on essential fatty acids and lipids in fish nutrition, see Tocher (2003).

7. Laboratory Activities

7.1 General Principles

Working with live fishes under laboratory conditions requires attention to many details concerning the requirements for, and limits of tolerance of, the particular species under study. Acceptable physical facilities and an adequate supply of water with good quality must be provided, even if the fishes are to be held for only 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 fishes are held.

The reader should note that some content of section 7 is not restricted to laboratory activities, but may be applicable to field situations, as well.

7.2 Confinement, Isolation, and Quarantine

Prior to bringing fishes into a laboratory, facilities and plans should be in place to ensure that the fish cannot escape, 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 or another disinfectant such as Virkon® Aquatic (www.wchemical.com/). 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 (see section 2.5 Fish Health Management: Control of Pathogens and Parasites).

Individual fish with suspected ill health should be quarantined from the others so as to negate the potential for spread of potential disease agents. Such fish should be evaluated by an individual with expertise in fish diseases (fish pathologist or veterinarian), and the proper therapeutant should be applied as directed. Providing guidance for the treatment of specific diseases is beyond the scope of this document. The investigator is strongly urged to establish a working relationship with individuals with expertise in fish health with whom they may consult.

Experimentation with nonindigenous fishes, transgenic fishes, or other genetically modified fishes is a special situation that requires additional precautions to preclude their escape. Permitting with site visits by state wildlife agencies may be required for holding nonindigenous species (see section 3.4 Permits and Certificates). 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 research activities. These USDA guidelines (1995a, 1995b) 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 locations, construction of containment structures, and nonstructural containment strategies. Institutional guidelines for working with transgenic or other genetically modified animals must be variable enough to adapt to site-specific and study-specific goals but should be sufficient to ensure that accidental release cannot occur during floods or other natural disasters or during equipment failures. Ultimately, individual scientists are responsible for ensuring the containment of animals.

Effluents from units used to hold newly introduced fishes should be treated. At a minimum, effluents should pass through screens with openings sufficiently small to retain any escaped fishes and, in turn, chemical or other treatments should be applied to kill all pathogens and parasites if they are expected to be present. Facilities conducting research on controlled disease agents (see OIE lists at http://www.oie.int/our-scientific-expertise/specific-information-and-recommendations/animal-disease-information/) must have isolated, self-sufficient units for the conduct of the research and must restrict access of unauthorized individuals to these units. (See Appendix Table 2 for a list of OIE-notifiable diseases in fish and amphibians.) 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).

Many common fish pathogens are opportunistic and are present in virtually all environments. Some are difficult to avoid (e.g., *Flavobacterium columnare*, the causative agent of columnaris disease); others are obligate pathogens and can only survive for a limited period of time if they are not in the host fish species (e.g., Viral Hemorrhagic Septicemia Virus [VHSV] Genotype IVb in the Great Lakes and the St. Lawrence River). While the investigator can reduce problems from opportunistic pathogens by using good husbandry, the obligate pathogens must be avoided. This can be typically done by establishing an integrated fish health management plan with regular fish health inspections by appropriately qualified fish health professionals. The investigator must be aware of certain diseases and agents that are problematic in the specific geographic region where work is conducted (e.g., VHSV IVb) and should be keenly aware of newly emerged diseases. The USDA National Invasive Species Information Center is a resource for specific microbes (http://www.invasivespeciesinfo.gov/microbes). Regardless, implementing biosecurity protocols is an effective strategy to minimize, if not eliminate, the risk of spreading localized or ubiquitous pathogens and invasive species. Thus, biosecurity (see section 3.2 Biosecurity) is an important consideration for both field and laboratory studies, whether or not strict biosecurity protocols are specifically mandated (e.g., when dealing with OIE-notifiable fish

pathogens including or injurious fish species; see Appendix Table 2 and USFWS information on injurious wildlife, http://www.fws.gov/injuriouswildlife/).

Comprehensive biosecurity plans can go well beyond simple disinfection procedures to include information on a wide variety of topics such as holding facility layout and design, fish sourcing and quarantine, and record keeping (see section 3.2 Biosecurity). What constitutes effective biosecurity will vary from one setting or research scenario to another. The Guide to Using Drugs, Biologics, and Other Chemicals in Aquaculture (AFS Fish Culture Section 2011, http://www.fws.gov/fisheries/aadap/AFS%20FCS%20Guide%20to%20Drugs.htm) offers additional information and resources related to disinfection and biosecurity practices. Included in this document is a table summarizing the various disinfectants (e.g., iodine, alcohols, chlorine) that may be used on field gear and hard surfaces that may come into contact with aquatic animals (AFS Fish Culture Section 2011).

7.3 Acclimation to Laboratory Conditions

Before studies begin, fishes should be given time to acclimate to new environments (see section 5.8 Field Acclimation), feeds, and routine activities. Slow acclimation to change is often critical (Casebolt et al. 1998). It is not uncommon for fishes to exhibit acute health problems 48–72 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 1–2 weeks.

For alleviating concerns about microbial agents at the time of fish transfer to the laboratory, formalin can be applied at 25 mg/L as a prolonged bath with additional aeration to the holding system. Formalin increases the chemical oxygen demand during decomposition over the 12–24 hours after administration. The formalin bath is commonly repeated at weekly intervals for 3 weeks during the initial fish holding period. Salt is another compound that can be used. If eggs or eyed eggs are brought from the field into the lab, other disinfectants such as iodine, hydrogen peroxide, or formalin can be used. Investigators can refer to the Blue Book (AFS–FHS 2012) for specifics on disease agents, fish species, and suggested methods.

Investigators should note that laboratory holding conditions may cause physiological changes in animals brought from the field. Even though no visual signs of stress may be present, immunosuppression (Miller and Tripp 1982) or loss of tidal or diel rhythmicity may occur. (See section 4.2 Stress.)

7.4 Facilities for Long-Term Housing of Fishes

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. Often, systems are arranged as flow-through systems, with a constant flow of fresh ("single-pass") water; however, well-designed and appropriately operating recirculation systems can maintain water of adequate or even superior quality (Malone and Beecher 2000; see section 7.8 Water Recirculation Units).

Culture systems will vary according to the physical size of the lab, the availability of water, the fish species, the number and density of test animals (see section 7.5 Density of Animals), and cost considerations. Fishes can be raised and maintained successfully in many types of systems, but there are optimum conditions for each species (see section 5.7 Facilities for Temporary Holding and Maintenance). In the design, minimizing stress should be a factor paramount for ensuring quality research animals. Adequate water flow providing both volume and flow patterns will deliver adequate dissolved oxygen and flush metabolic waste products (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 items such 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; see section 7.2 Confinement, Isolation, and Quarantine). Implementing pathogen control measures is an emphasis for fish biomedical research facilities because underlying disease or chronic infections can impact host physiology and other research endpoints (Lawrence et al. 2012).

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 section 7.7 Water Quality). (Suboptimal water quality and contaminating compounds can influence physiological parameters and behavior). Floor drains should be numerous and appropriately spaced; floors need not be impervious to water but should be slip-resistant. Ground-fault interrupted electrical connections will assure animal and personnel safety. Log books for equipment checks and maintenance, and for animal feeding and record-keeping are typically in use in housing facilities. Construction materials are available that minimize contact with potentially toxic substances. Appropriate construction materials for the holding system components (e.g., tanks, valves, delivery lines, and drains) include glass, type 316 stainless steel, nylon, fluorocarbon plastics, concrete (ASTM 2013), polyethylene sheeting, rigid PVC, Teflon®, and fiberglass (U.S. Army Corps of Engineers 1991). Brass, copper, lead, zinc, and rubber should be avoided (ASTM 2013), as should corrodible substances (Hawkins 1981). Regular monitoring of water quality is essential (see section 7.7 Water Quality). 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.

7.5 Density of Animals

The density of fish that can be held in an experimental unit depends on a series of environmental factors. The most immediate issues are maintaining a supply of dissolved oxygen and the water temperature and elevation (Piper et al. 1982). Accumulation of waste products, especially ammonia, is generally the next factor limiting density (Piper et al. 1982). Oxygen demand and excretion of ammonia are directly related to the amount of feed supplied to the fishes. 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 static units because of continual replenishment of dissolved oxygen and removal of ammonia. However, bead filter technologies used in recirculation systems have increased fish densities that can be maintained (Malone and Beecher 2000; see section 7.8 Water Recirculation Units). Static units must be equipped with aeration and charcoal filtration equipment if the density of fishes is greater than the minimum 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 mg/L. Ammonia concentrations should be near zero, especially at higher pH levels (see section 7.7 Water Quality). Physiological stress, susceptibility to disease agents, and transmission of disease agents are additional factors that must be considered when density levels are established.

Fish vary from species to species, and even within a species, as to the degree of crowding that they will tolerate before 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). Generally, practical density is determined by the water treatment and feed delivery systems and reaches its maximum at that density as 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 the variability in appropriate densities for various species and specific studies. No standard, preferred density applies to all species.

7.6 Feeds and Feeding

Although most species of adult fishes can survive several weeks without food (especially at lower temperatures), they must be provided with food that is palatable and meets basic nutritional requirements in order to remain in satisfactory condition as research subjects. Migratory fishes or other fishes which exhibit fasting as a normal part of their life cycle or behavior may be exceptions to this general rule. A review of a species life history can provide guidance regarding feeding requirements. If the nutritional requirements 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. It cannot be assumed that supplying natural foods, especially of a single type, will meet the complete nutritional requirements of the captive fishes. Some species can be trained with relative ease to accept formulated feeds, thereby eliminating the problems inherent in providing natural foods, such as high cost, inconvenience, and inadvertent pathogen introduction.

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 fishes frequently will consume feeds designed for other species (e.g., carnivorous fish will consume feeds developed for Rainbow Trout), their nutritional 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 fulfill the guaranteed analysis description provided with the feed can vary considerably from batch to batch, even though the proximate composition (protein, carbohydrate, fat) remains constant. Thus, a feed may meet overall macronutrient demands (e.g., protein), but optimal levels of specific nutrients (e.g., essential amino acids) may not always be met for each fish species. 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) and consider consulting with a feed manufacturer or fish nutritionist when selecting diet formulations. Switching diets can have effects on the experimental animals (Gatlin 2010; see Southern Regional Aquaculture Center fact sheets are available at https://srac.tamu.edu/index.cfm/event/viewAllSheets/).

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, and environmental conditions, especially water temperature. Feeding to satiation is the normal practice unless the research design or other logistics dictate reduced feeding rates. The weight of formulated feed to be fed, per manufacturers' instructions, is generally lower than that required for natural foods because formulated feeds have lower moisture content than do natural foods. Typically, formulated feeds are fed at levels ranging from 1% to 8% of the weight of the fish per day, depending on water temperature and on the species, size, and age of the fish. Optimal feeding times depend on species-specific behavior but generally can be modified to accommodate reasonable 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 themselves. The method of feeding combined with the stocking density may differentially impact behavior and stress (Attia et al. 2012; see section 7.5 Density of Animals). Feeding by machine has the potential to prevent habituation by the fish to the presence of feeding personnel and allows flexibility in feeding

schedules. Excess uneaten feed should be removed from a tank within a short time following feeding, as it decreases water quality and can support fungal growth. Water quality will be diminished by accumulated feed, and water-soluble nutrients will be leached from the water-soaked pellets.

7.7 Water Quality

Providing water of appropriate physical and chemical quality is probably the single most important 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, the investigator is responsible for determining the preferred conditions for the species under study. Transferring fishes into water having a temperature outside their limits of tolerance, or in excess of their capacity to adapt, can lead to death, either immediately or delayed, usually within 72 hours. Sudden changes in water temperatures as small as 5°C can cause stress responses in fishes that are otherwise healthy. Experienced investigators do not routinely expose fishes to temperature changes greater than 2°C per day. Limits of tolerance and ability to tolerate temperature changes are influenced by the previous thermal histories of individual fish as well as species characteristics (Carmichael et al. 1984a; Berka 1986).

The presence of toxic substances in water or the absence of sufficient dissolved oxygen can cause immediate death to fishes 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 fishes, water supplies used to hold fish should be analyzed for parameters such as hardness, alkalinity (buffering capacity), major cations (Na^{1+}, K^{1+}, Ca^{2+}, Mg^{2+}), major anions (CO_3^{2+}, Cl^{1-}, HCO_3^{1-},SO_4^{2-}), heavy metals, and pesticides. Routine, periodic monitoring of temperature, dissolved oxygen, ammonia, alkalinity, nitrite, and pH should be conducted. In the case of soft water, or water that is poorly buffered, substantial changes in pH may cause adverse effects. Un-ionized ammonia is quite toxic to fishes and a cause of stress or even mortality, especially at higher pH levels. The addition of buffering agents may be warranted in these 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).

Municipal water or any supply that has been chlorinated should be monitored regularly especially for free chlorine which can produce immediate toxic reactions. Dechlorinating equipment, such as activated charcoal filters and chemicals (e.g., sodium thiosulfate) are commercially available that can reduce free chlorine to undetectable concentrations. Even so, very low concentrations of chlorine byproducts or metabolites may remain in the water. Normally these chemicals do not cause short-term adverse effects, but the potential effects should be considered and their concentrations monitored if they could affect results.

Contaminants in water have the potential to impact physiological characteristics of fishes, and fishes are often used in ecosystem monitoring projects (see section 5.2.3 Representative Samples). For example, water downstream from sewage treatment plants and industrial sources can contain endocrine modulators or disruptors (e.g., Arukwe 2001; Kolpin et al. 2002; Jenkins et al. 2013). Increasing evidence indicates that drinking water itself may contain various personal care products and drugs, albeit at very low concentrations (Benotti et al. 2009).

7.8 Water Recirculation Units

The emergence of water recirculation systems in aquaculture over the past three decades has provided several benefits to fish culturists and investigators. The substantial water supply requirements and specific climatic conditions required by traditional fish culture systems are eliminated; fishes can be produced and maintained year-round, and environmental impacts of organic effluent discharges are reduced (see section 7.9 Effluents and Permits). Recirculation 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 recirculation 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 recirculation aquaculture systems. Additional parts may include pumps, tanks, clarifiers, aeration and oxygenation components, UV light sterilizers or ozonation generators, and sumps. Backup power supplies in case of power failure are assets.

Several types of biofilters are available. Those with the highest nitrification efficiencies function best to control ammonia and nitrite levels. (Nitrification, accomplished by bacteria in such genera as *Nitrosomonas*, *Nitrospira*, and *Nitrobacter*, is the process of ammonia removal and consists of successive oxidations of ammonia to nitrite and finally to nitrate.) In order to establish an active nitrifying bacterial population, the biological filter must be preconditioned for a period of several weeks prior to stocking with high fish densities. 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 shortening the startup time of a seawater system (Nijhof and Bovendeur 1990).

When recirculation systems are designed and operated, management plans should be developed to maintain the function of the system under unusual conditions such as disease outbreaks. Biofilter bacteria can be killed by therapeutic antibacterial agents and parasiticides (Heinen et al. 1995). Appropriate startup procedures and preconditioning are essential prior to introducing fishes into recirculation systems.

7.9 Effluents and Permits

Facilities holding fishes will produce wastewaters, and 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 municipal drainage systems or wetlands, but ultimately, they will move into a public water body. Most effluents from wet labs can be safely added to treatment plants or even public water bodies.

Regulatory authority and determination of acceptable effluent contributions rests initially with the EPA or an EPA-delegated authority such as a state authority (Hindrichs and Cormier 2009). Discharge of wastes or pollutants entering waters of the United States requires a National Pollutant Discharge Elimination System (NPDES, http://cfpub.epa.gov/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 EPA as concentrated aquatic animal production facilities according to their size and the type of fish produced (CFR 2000). 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 of fish per month, are classified as 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 of fish also need NPDES permits. Smaller aquaculture facilities may need permits. Investigators conducting tests in aquaculture facilities where an undeclared pollutant, such as a new drug treatment, might be discharged need to contact the EPA or its designee to determine safety. Often, the facility operator will need to amend their Notice of Intent (NOI) to be covered under a particular NPDES permit to include the new drug in their discharge (see section 7.11 Restraint of Fishes: Sedatives and Related Chemicals). Failure to secure discharge approval prior to discharge results in a violation of the Clean Water Act (United States Code 2002) and can result in substantial fines and incarceration.

7.10 Dangerous Species and Specimens in Captivity

In addition to the recommendations provided in section 5.5 Dangerous Species and Specimens, investigators 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 and personal protective equipment that address the specific problems related to each species, and individuals should never work alone. Prior to the start of an investigation or housing dangerous species, a formal job hazard analysis must be developed. This analysis is a cooperative effort among the principal investigator, the institute's Environmental Health and

Safety Officer, and the IACUC, specifically with consultation of the IACUC's veterinarian. This job hazard analysis (see https://www.osha.gov/Publications/osha3071.html) will take into account any information that might be available from industry and/or federal government agencies familiar with these issues, species, and specimens.

7.11 Restraint of Fishes: Sedatives and Related Chemicals

Prolonged, stressful restraint should be avoided. In some cases, general sedation for restraint may be advisable (Mundy and Wilson 1997); however, the benefits of sedation and potential effects on data derived from sedated fishes might need to be compared to results obtained from fishes that have not been sedated. The full range of potential effects on the subject fish, not just the sedative qualities, must be considered. The sedative chosen should be one that permits a rapid return to normal physiological and behavioral status (Smith et al. 1999; Bowker and Trushenski 2011) and is a low-risk compound for humans, as well as fishes. The compound should be tested on a small sample of fish prior to widespread use. Sedated animals must be kept under observation until appropriate recovery occurs.

The following substances have been used by various investigators (some are controlled substances available only through appropriately licensed sources, such as veterinarians): benzocaine, clove oil, diazepam (valium), sodium pentobarbitol, and tricaine methanesulfonate (MS-222). Hypothermia and exposure to sublethal levels of CO_2 (an LRP drug; see section 5.4.2 Low Regulatory Priority (LRP) Drugs and Appendix Table 1) have been used in situations where other sedatives were contraindicated. The only sedative approved by the FDA for general use on fishes is MS-222, but a 21-day withdrawal period is required before the treated fish may be released or consumed by humans.

The FDA allows investigators some degree of choice in the selection and use of drugs, including sedatives, if the fish use is research only and not for consumption or release. Strict interpretation of FDA policies would allow such choice only for approved studies on drugs where discharge of the drug is in compliance with local NPDES permits; however, enforcement practices typically allow greater flexibility (see section 7.9 Effluents and Permits). The FDA Center for Veterinary Medicine (CVM, http://www.fda.gov/AnimalVeterinary/default.htm) or the USFWS AADAP Program (http://www.fws.gov/fisheries/aadap/home.htm) may be contacted to determine current practices and priorities.

The complexities related to FDA drug approvals and the experimental use of drugs in research are illustrated by recent actions related to clove oil. Clove oil, in any form, may not be used on fishes that could 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. At the time of this writing, only CO_2 (as

an LRP drug; see Appendix Table 1) and eugenol (AQUI-S® 20E; under a USFWS INAD exemption) can be used as immediate-release sedatives (see section 5.4 Field Restraint of Fishes: Sedatives). Alternatively, electrosedation or electroanesthesia may be suitable alternatives to chemosedation when fishes need to be sedated multiple times, when discharge of the chemosedative is not feasible, or when the fishes need to be released immediately into the environment (Trushenski et al. 2012a, 2012b).

7.12 Surgical Procedures

Surgical procedures, written into many research plans, include such processes as implanting devices including tags and transmitters, examining internal organs (e.g., gonads), and removing organs. Successful surgery depends on the complexity of the procedure, the expertise of the individual, and the environment in which the procedure is conducted. To perform surgeries on fish, individuals require training and practical experience. Generally, fisheries biologists lack formal educational training in surgery, and as such, training should be provided by individuals with long-standing experience, extensive surgical expertise, and a record of surgical success with fishes. Formal training opportunities offered at institutions would benefit researchers proposing to use surgical procedures. Individuals suited to train may be veterinarians with experience with fishes and who are associated with the IACUC, a member of the veterinary group providing health oversight to animals (including fish) at an institution, or an investigator with extensive experience in the successful performance of surgical procedures with fish. The training would cover the principles of surgery and guidance in performing the specific procedure. At the end of the training, the trainee would demonstrate to the instructor that they are capable of performing timely and effective surgeries similar to the procedures proposed for their research.

Written protocols for proposed surgical procedures should clarify whether the surgery is considered a major surgery or minor surgery. Major surgery is a procedure that penetrates or exposes a body cavity, or a surgery that produces a substantial physical or physiological impairment. Examples of major surgery are implantation of a radio transmitter in the coelomic cavity, or a splenectomy. Minor surgery is less invasive, not penetrating or exposing a body cavity or producing substantial physical or physiological impairment. Examples of minor surgery include placement of catheters in blood vessels or implanting PIT tags in the muscle (see section 6.3 Internal Tags and Marks, and Biotelemetry).

The aquatic nature of fish necessitates surgical procedures not considered customary with non-aquatic animals. Body surfaces of fish are not covered by a keratinized integument but rather by a variety of living cells and mucus that provide a physical and immunological external barrier. Because many disinfectants can damage fish skin, the complete disinfection of a surgical site is likely not possible (Stoskopf 1993c); the preparation of the surgical site generally consists of gently cleaning the area of excess mucus. Efforts to avoid contaminating the surgical field and postsurgical infections are very important.

Realistic, pragmatic issues of the logistics for asepsis must be considered in the development of surgical protocols that are to be performed in the field in contrast to the well-equipped and designed laboratory or surgical suite. The ability to employ all of the aseptic techniques of surgery (e.g., use of gown, mask, drape) is influenced by the environment in which the surgery is performed. Whether the type of surgery is considered a major or a minor procedure is especially relevant when high numbers of fish will undergo surgery that must be performed at remote field locations. Reports have documented investigations of techniques that support successful surgery on fish under non-laboratory conditions (Jepsen et al. 2013). These reports documented what could be considered reasonable deviations in surgical procedures that still result in surgical success. The statement in NRC (2011: 115), "Modification of standard techniques may be required (for instance, in aquatic or field surgery), but should not compromise the well-being of the animals," intimates that the primary investigator be pragmatic.

Investigators should review published studies for relevance to the procedures they propose to perform. One area where a deviation from standard surgical procedures may be appropriate is that of sterilization of instruments between fish. In remote field locations, where high numbers of fish are to be processed, it may not be possible to access an autoclave or other standard sterilization equipment. In such cases, instrument disinfection between fishes might be performed by immersing the instruments in a 1:1,000 solution of benzalkonium chloride or other quaternary ammonia compound (Summerfelt and Smith 1990) and 70% ethanol and then rinsing the instruments in sterile water before use. Other options for processing surgical instruments between surgeries are hot bead sterilizers or ultraviolet systems (Walker et al. 2013). Combinations of methods may be appropriate. In all cases, the surgical team should strive to the best of their ability to avoid contamination of the surgical field.

Depending on the anesthetic used (i.e., MS-222) and whether the fish will be stressed during a post-surgery holding period, temporary confinement may be necessary for a withdrawal time prior to their release into the environment, as they may be caught and consumed by the sport fishing community (see section 5.4.1 Drugs Approved for Use on Fish). Because of the diversity of surgical procedures that may be performed on fish, the information presented in the Guidelines is not intended to provide a detailed guide to fish surgery. Additional details may be found in Deters et al. 2010, Stoskopf 1993c, Summerfelt and Smith 1990, Smith and Bell 1967, and Wooster et al. 1993. Information on surgical implantations is found in Liedtke et al. 2012, Chomyshyn et al. 2011, Cooke et al. 2011, Brown et al. 2011, and Wagner et al. 2011 (see section 6. Marking and Tagging).

Generally, listed, imperiled species must be handled noninvasively, and if surgery is to be done, a permit is needed (see section 5.2.4 Collection of Imperiled Species).

7.13 Administration of Drugs, Biologics, and Other Chemicals

Research investigators using fish drugs, biologics, and other chemicals are responsible for following federal, state, and local regulations. Careful records are to be maintained for documenting each use.

7.13.1 Drugs

The Federal Food, Drug and Cosmetic Act (FD&C Act 2013, http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/default.htm) defines drugs, in part, by their intended use, as "articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease" and "articles (other than food) intended to affect the structure or any function of the body of man or other animals." According to these definitions, virtually any product administered to a fish is considered a drug. All drugs used to control mortality associated with bacterial diseases or infestation density of parasites, sedate or anesthetize fishes, induce spawning, change gender, or change the structure or function of aquatic species must be approved by the FDA. Ice, an innocuous compound, is therefore considered a drug as it slows metabolic rates, and salts are considered drugs because of the influence on osmoregulation. The FDA approves drugs as compounds for which data have been evaluated by CVM (Appendix Table 1) and for which they conclude are effective in achieving the stated claim, including being safe to the target fishes, to humans who either handle the active ingredient or consume the treated fish, and to the environment when applied at labeled doses. The drugs must be manufactured according to FDA CVM criteria and be packaged and labeled in a manner to ensure use compliance. The FDA can require investigators to provide thorough records of drugs used.

Currently, there is no drug that is "conditionally approved" for use on fish. A conditional approval allows a drug to be legally marketed prior to the completion of data collection and acceptance by the FDA. Conditional drug approvals mean that the compound appears safe, is being manufactured according to FDA CVM criteria, and shows effectiveness; however, the compound is generally not widely available. Illegal drug use for fish includes (1) use of unapproved drugs for any purpose or (2) use of approved drugs in a manner other than that specified on the product label (unless listed on INAD exemption or unless prescribed for extra-label use by a licensed veterinarian). Interstate transport of drugs for unapproved use in any animal, including fish, is prohibited. Additional information on approved and investigational new animal drugs can be obtained at the AADAP (http://www.fws.gov/fisheries/aadap/home.htm) or FDA CVM (http://www.fda.gov/AnimalVeterinary/default.htm) Web sites. A provision allows for the use of drugs in animals in teaching settings, as described in CFR 1976.

Licensed veterinarians have the authority to prescribe extra-label (see section 2.5 Fish Health Management: Control of Pathogens and Parasites) uses for drugs. Some chemicals can be administered only through veterinary cooperation (e.g., Chorulon®, veterinary feed directive drugs).

7.13.2 Biologics and Other Chemicals

Use of "biologics" with fishes generally refers to vaccines, bacterins, antisera, diagnostic kits, and other products of biological origin. These veterinary and research products are used to diagnose, prevent, or treat animal diseases, and a number of licensed, commercial biologics are approved for use in fish. For use in aquaculture, see USDA APHIS Program Aid No. 1713 Veterinary Biologics: Use and Regulation (USDA 2013, http://www.aphis.usda.gov/publications/animal_health/content/printable_version/vet_biologics.pdf) and Use of Vaccines in Finfish Aquaculture (Yanong 2011, http://edis.ifas.ufl.edu/fa156), and for more information see USDA APHIS Center for Veterinary Biologics (CVB) Web site (http://www.aphis.usda.gov/wps/portal/banner/help?1dmy&urile=wcm%3Apath%3A/APHIS_Content_Library/SA_Our_Focus/SA_Animal_Health/SA_Vet_Biologics), which regulates products, and the AFS Fish Culture Section Guide to Using Drugs, Biologics, and Other Chemicals in Aquaculture (AFS Fish Culture Section 2011, http://www.fws.gov/fisheries/aadap/AFS%20FCS%20Guide%20to%20Drugs.htm).

7.13.3 Chemical Facility Anti-Terrorism Standards (CFATS)

Certain aquaculture drugs may present security issues if they are released, stolen, or diverted or used for purposes of sabotage or intentional contamination. Facilities storing these chemicals may be regulated by the Department of Homeland Security (DHS, http://www.dhs.gov/) under the Chemical Facility Anti-Terrorism Standards 2007 (CFATS, http://www.dhs.gov/chemical-facility-anti-terrorism-standards). Facilities that manufacture, use, store, or distribute certain chemicals at or above a specified quantity may be regulated. Each facility is responsible for evaluating their own chemical use patterns and determining which are subject to CFATS. The DHS has identified more than 200 chemicals of interest (Federal Register 2007, http://www.dhs.gov/xlibrary/assets/chemsec_appendixa-chemicalofinterestlist.pdf); some of special concern to fisheries research facilities include

- Formalin/formaldehyde solution—when greater than or equal to 1% solution and greater than or equal to 15,000 pounds are stored.
- Hydrogen peroxide—when greater than or equal to 35% solution and greater than or equal to 400 pounds are stored.
- Potassium permanganate—when commercial grade and greater than or equal to 400 pounds are stored.

Facilities may be required to develop a Site Security Plan if it is determined to be high risk by DHS (www.dhs.gov/chemicalsecurity).

8. Final Disposition of Experimental Animals

8.1 Euthanasia

Several methods are available to euthanize fishes. Various regulatory or granting agencies may require specific euthanasia methods and written protocols that demonstrate sufficient attention to humane treatment. Some methods for euthanasia are listed by American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition (AVMA 2013, https://www.avma.org/KB/Policies/Documents/euthanasia.pdf), and general considerations in the Guide for the Care and Use of Laboratory Animals (NRC 2011).

In general, the procedures must be performed quickly and with minimal stress prior to dispatch. Spinal cord dislocation, or decapitation generally are acceptable methods, provided the procedure is performed quickly and accurately. Some IACUCs have approved protocols where small fish (<5 cm total length) may be euthanized by cold stunning in an ice bath (Wilson et al. 2009; Blessing et al. 2010). Small fishes may be euthanized instantly by immersion in liquid nitrogen following sedation (Schaffer 1997); however, this approach and its appropriateness should be discussed with an IACUC or other oversight body prior to use. 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, where fish may be considered commodities rather than research specimens. Small numbers of fishes can be euthanized by exposure to relatively high concentrations of sedatives such as MS-222; however, the use of MS-222 (AVMA 2013, https://www.avma.org/KB/Policies/Documents/euthanasia.pdf) and other chemical sedatives as euthanizing agents has not been approved by the FDA, and these chemically euthanized fishes may not be made available for human or animal consumption. See section 7.11 Restraint of Fishes: Sedatives and Related Chemicals. See Coyle et al. (2004) on anesthetic use with aquatic animals, including dosages used with commonly cultured fish species (Southern Regional Aquaculture Center fact sheet 3900 at https://srac.tamu.edu/index.cfm/event/getFactSheet/whichfactsheet/162/). Euthanasia through simple oxygen deprivation (dewatering) 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 is a suggested alternative if large numbers of fishes must be euthanized. Rotenone, which blocks oxygen uptake, has been useful in cases of the occurrence of unintended exotics (Rayner and Creese 2006) (see section 5.2.3 Representative Samples). Selection of euthanasia methods should be done in coordination with the institutional IACUC. Additional information is provided by The University of Florida Extension Service, where publications addressing methods of fish

slaughter, killing, and euthanasia are reviewed (Yanong et al. 2007, http://edis.ifas.ufl.edu/pdffiles/FA/FA15000.pdf).

Marine fish surveys conducted at sea present a special set of conditions with respect to euthanasia. The capture methods tend to result in substantial numbers of specimens collected at one time. Information on sex, sexual maturity, and stomach contents may be obtained from individuals 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 at each station or sampling event. Euthanasia of individuals 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, investigators and agencies may be granted exemptions from standard practices for euthanasia.

8.2 Storage or Return to Aquatic Habitat

Applications for animal use submitted to IACUCs typically require the principal investigator to delineate the disposition of fish or fish carcasses. Whether required or not, options for final disposition of experimental subjects should be delineated in research study plans. Fish collected in the wild and brought into the laboratory for experimental or teaching purposes should never be returned to the environment according to the general consensus among agencies granting scientific collecting permits. After proper euthanization and preservation, study animals may be again useful as teaching- or voucher specimens in research collections (see section 5.2 Field Collections). Federal, provincial, state, or local laws may prohibit release of study animals under any circumstances. Nonnative fishes should never be released. In some cases, transfer to another research project or educational exercise may be an appropriate disposition, and permitting may be required in the case of federally listed specimens. If appropriate SOPs for disposition are available, they could be followed, in addition to relevant guidelines established by the research institution and local government.

9. Future Revisions

Periodic revisions (approximately every 5–8 years) of the Guidelines will be necessary and best addressed by a multidisciplinary committee. Although the basic philosophies of the scientific methods 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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Appendix

Brief Checklist for IACUC Readiness

The following checklist provides a quick reference of factors that investigators should consider in preparing plans for Institutional Animal Care and Use Committees (IACUCs) (see section 2.1 Approval of Research Plans by IACUCs). 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.

- Choice of Taxa
- Number and Choice of Individuals
- Literature Search
- Population and Genetic Considerations
    - Captive/Domestic Stocks
    - Wild Stocks
    - Threatened or Endangered Species
- Animal Welfare
- Living Conditions and Acclimation
- Water Quality
- Foods, Feeds, and Feeding
- Health
- Stress
- Emergency Preparedness
- Sedation
- Euthanasia
- Permits and Regulations

List of Low Regulatory Priority Drugs and Consideration for Their Use

Although technically unapproved for use in fishes, low regulatory priority (LRP) drugs (Appendix Table 1) are compounds that the U.S. Food and Drug Administration Center for Veterinary Medicine (FDA CVM) considers to be of comparatively little risk to aquatic organisms, human consumers, or the environment. The FDA CVM has stated that it is unlikely to regulate the use of LRP drugs if the following five conditions are met: (1) the substances are used for the listed indications, (2) the substances are used at the prescribed levels, (3) the substances are used according to good management practices, (4) the substances are an appropriate grade for use in food animals, and (5) there is not likely to be an adverse effect on the environment.

Appendix Table 1. Low regulatory priority aquaculture drugs, indications, and doses.

Compound	Indication(s)	Dose
Acetic acid	Parasiticide for fish	1,000–2,000 ppm dip for 1–10 min
$CaCL_2$	Used to aid in egg adhesion Used to aid in maintaining osmotic balance during fish holding and transport	10–20 ppm $CaCO_3$ (eggs) ≤150 ppm $CaCO_3$, indefinitely (fish)
CaO	External protozoacide for fish	2,000 ppm dip for 5 sec
CO_2 gas	Anesthetic for fish	
Fuller's earth	Used to reduce the adhesiveness of fish eggs	
Ice	Used to reduce the metabolic rate of fish during transport	
$MgSO_4$	Used to treat external monogenic trematode or crustacean infestations in fish	30,000 ppm $MgSO_4$ + 7,000 ppm NaCl dip for 5–10 min
Papain	Used to remove the gelatinous matrix from fish egg masses	0.2% solution
KCl	Used to aid in osmoregulation, relieve stress, and prevent shock of fish	10–2,000 ppm KCl
Povidone iodine	Egg surface disinfectant	100 ppm for 10 min during or after water hardening
$NaHCO_3$	Used to introduce CO_2 into water to anesthetize fish	142–641 ppm for 5 min
NaCL	Used as an osmoregulatory aid to relieve stress and prevent shock in fish Parasiticide for fish	0.5%–1.0% indefinitely 3% dip for 10–30 min
Na_2SO_4	Used to improve hatchability of fish eggs	15% solution for 5–8 min
Thiamine hydrochloride	Used to prevent or treat thiamine deficiency in salmonids	≤100 ppm for ≤4 h during water hardening ≤1,000 ppm for ≤1h (sac-fry)
Urea or tannic acid	Used to reduce the adhesiveness of fish eggs	Immersion in 3 ppt urea + 4 ppt NaCl for ~6 min followed by separate immersion in 150 ppm tannic acid for ~6 min (treats approximately 4,000,000 eggs)

Appendix Table 2. OIE-notifiable causative disease agents for fish and amphibians.

Fish disease agents

Aphanomyces invadans (fungus)

Gyrodactylus salaris (parasite)

Epizootic haematopoietic necrosis virus

Infectious haematopoietic necrosis virus

Infectious salmon anaemia virus

Koi herpes virus

Red sea bream iridovirus

Spring viraemia of carp virus

Viral haemorrhagic septicaemia virus

Amphibian disease agents

Batrachochytrium dendrobatidis

Ranavirus

Office International des Epizooties publishes their current list online in the Aquatic Animal Health Code http://www.oie.int/index.php?id=171&L=0&htmfile=titre_1.10.htm

Index of Terms and Acronyms

AADAP (Aquatic Animal Drug Approval Partnership Program) 5, 29
AFS (American Fisheries Society) ..... ix, xi, 2
AIFRB (American Institute of Fishery Research Biologists) ..... ix, 2
APHIS (Animal and Plant Health Inspection Service) 15, 56
ASIH (American Society of Ichthyologists and Herpetologists) ..... ix, 2
AVMA (American Veterinary Medical Association) 59
CFATS (Chemical Facility Anti-Terrorism Standards) 56
CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) 9, 14
DHS (Department of Homeland Security) 56
DNA (deoxyribonucleic acid) 40, 41
EPA (U.S. Environmental Protection Agency) 4, 5, 8, 51
FDA (U.S. Food and Drug Administration) 4, 5, 7, 8, 29, 30, 39, 52, 55, 59
FDA CVM (U.S. Food and Drug Administration Center for Veterinary Medicine) 52, 55, 85
IACUC (Institutional Animal Care and Use Committee) ..... xiii, 2, 3, 4, 13, 17, 18, 22, 47, 52, 53, 59, 60, 85
INAD (Investigational New Animal Drug) 8, 30, 53, 55
LRP (low regulatory priority) 8, 29, 52, 53, 86
MS-222 (tricaine methanesulfonate) 27, 29, 52, 54, 59
NIH (National Institutes of Health) 2
NOAA (National Oceanic and Atmospheric Administration) 25, 26
NPDES (National Pollutant Discharge Elimination System) 51, 52
OIE (World Organisation for Animal Health [formerly Office International des Epizooties]) 9, 12, 44, 76
PHS (Public Health Service) 2, 12, 13
PIT (passive integrated transponder) 38, 39
PQAP (Project Quality Assurance Plan) 4, 5
SOP (standard operating procedure) 4, 5, 30
SRAC (Southern Regional Aquaculture Center) 29, 33, 48, 59, 90
USDA (U.S. Department of Agriculture) 12, 13, 43, 44, 56, 81
USDHHS (U.S. Department of Health and Human Services) 2
USFWS (U.S. Fish and Wildlife Service) 5, 14, 25, 26, 52, 53

Note on Additional Readings

The 2004 version of the Guidelines (Use of Fishes in Research Committee 2004) listed particular citations under topics that would add to the understanding and background on those topics. This UFR Committee is suggesting that more current information may be obtained on uses of fish in research by conducting online literature searches. Additionally, relevant resources may be found at sites such as that of the Southern Regional Aquaculture Center (https://srac.tamu.edu/index.cfm/event/viewAllSheets/).