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Development of Spore Protein of *Myxobolus koi* as an Immunostimulant for Prevent of Myxobolusis on Gold Fish (*Cyprinus carpio* Linn) by Oral Immunisation

Adapun penelitian ini sudah mengacu pada prosedur pertimbangan etik dari *American Fisheries Society* (AFS, 2014) yang berjudul *Guidelines for the Use of Fishes in Research* dan *Canadian Council on Animal Care* (CCAC, 2005) yang berjudul *Guidelines on the Care and Use of Fish in Research, teaching and Testing*. Sehingga penelitian tersebut tidak perlu dilakukan **Uji Ethical Clearance** karena menggunakan ikan yang minimal dan menghasilkan *out put* yang sangat baik.

Demikian Surat Keterangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar** atas nama Dr. Gunanti Mahasri, Ir., M.Si.

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Guidelines for the Use of Fishes in Research

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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/>).

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).

Canadian Council on Animal Care



guidelines on:
the care and use of
fish in research,
teaching and
testing

This document, the CCAC *guidelines on: the care and use of fish in research, teaching and testing*, has been developed by the *ad hoc* subcommittee on fish of the Canadian Council on Animal Care (CCAC) Guidelines Committee.

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the care and use of fish in research, teaching and testing



A. PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer review agency responsible for setting and maintaining standards for the care and use of animals used in research, teaching and testing throughout Canada. In addition to the *Guide to the Care and Use of Experimental Animals*, vol. 1, 2nd ed., 1993 and vol. 2, 1984, which provide the general principles for the care and use of animals, the CCAC also publishes detailed guidelines on issues of current and emerging concerns. The CCAC *guidelines on: the care and use of fish in research, teaching and testing* is the seventh of this series. This document supersedes Chapter I - Fish, *Guide to the Care and Use of Experimental Animals*, vol. 2 (CCAC, 1984).

These guidelines aim to provide information for investigators, animal care committees, facility managers and animal care staff that will assist in improving both the care given to fishes and the manner in which experimental procedures are carried out.

The present document has drawn substantially from the work of organizations listed in Appendix A. Their contributions to the development of these guidelines are gratefully acknowledged.

The guidelines have been developed by the CCAC subcommittee on fish and were reviewed by a total of 69 experts. A preliminary first draft was agreed on by the subcommittee and circulated to experts in June 2002 (including representatives of the organizations listed in Appendix A), and a second draft was circulated for widespread comment in June 2003. A final review was carried out in August 2004 involving all individuals who had previously provided significant input to the development process. The development of these guidelines also involved consultation with the Canadian Association for Laboratory Animal Science (CALAS) and the Canadian Society of Zoologists (CSZ) through workshops held at annual meetings in Québec City (June 2003), Acadia University (May 2004), and Hamilton (June 2004). Consultations were also held at the Aquaculture Association of Canada and AquaNet annual meetings in Québec City (October 2004), and at the CCAC Workshop on the Fish Guidelines in Vancouver (April 2005).

The guidelines have been organized in a format that should facilitate easy access to relevant sections. Early sections provide an ethical overview relevant to the use of fishes in research, teaching and testing. This is followed

by a brief overview of regulations and responsibilities relevant to the care and use of fishes in science in Canada. The remainder of the document provides guidelines to assist in caring for fishes in laboratory facilities, followed by guidelines to help in the development and review of experimental protocols. An overview of the CCAC *guidelines on: the care and use of fish in research, teaching and testing* is provided through a summary of the guidelines listed in

this document prior to the beginning of the main text.

The refinement of animal care and use guidelines is a continuous process. These guidelines are intended to provide assistance in the implementation of best practices, and should not be viewed as regulations. Where regulatory requirements are involved or where it is absolutely imperative to adhere to a particular guideline, the term *must* has been used.

B. INTRODUCTION

The greatest challenge in providing *guidelines on the care and use of fish* is the wide variety of fishes used in Canada and the diversity of their habits, behavior, life history, and environmental and husbandry requirements. In addition, the scientific information required to define the preferred conditions for fish well-being is limited. While considerable research has been conducted on culture strategies and environmental and water quality requirements, such studies have generally been aimed at determining conditions that optimize production in aquaculture systems, rather than improving the welfare of fishes, and have not usually addressed the difference between *tolerance* and *preference* (Fisher, 2000).

An important consideration in these guidelines is the naturally high mortality rates of juveniles in species whose ecological strategies include the generation of large numbers of progeny to ensure adequate survival in the wild. In addition, many experimental populations of species with usually high survival contain individuals that will not thrive to adulthood even under the best environmental conditions. In some situations, a population-based (or a group of study fish) approach to well-being may be appropriate, but individuals that are not likely to thrive should be euthanized as soon as they are identified.

Another consideration for these guidelines is the general acceptance by the public of the current killing methods used in harvesting wild fishes or in recreational angling. In general, the public appears to be willing to accept these killing methods for food production but not when fishes are used for research. These guidelines accept that for research, teaching, and testing use of any animal, including fishes, more emphasis will be placed on individual well-being than is generally accepted for the commercial harvesting or production of animals for food. It is recognized, however, that in some instances investigators may obtain fishes from people involved in commercial or recreational harvesting and have little influence over the capture methods.

These guidelines apply to fishes held in facilities for research, teaching and testing, as well as to fishes that are studied in their natural habitats.

1. Definition of Fish

For the purpose of these guidelines, fishes are defined as all bony and cartilaginous fish genera (classes Chondrichthyes [cartilaginous fishes], Agnatha, and Osteichthyes [bony fishes]). Fish eggs, embryos or larvae that have not developed beyond exclusive reliance on their own yolk nutrients are not covered by these guidelines. Similarly, invertebrates (except cephalopods) are not covered under the CCAC system of surveillance, but institutions are encouraged to foster respect for these animals by ensuring that holding facilities and levels of husbandry meet standards equivalent to those used for fishes.

2. Rationale for Guidelines on the Care and Use of Fish

The use of fishes as experimental subjects has increased substantially over the past two decades. This increase in use is a result of the rapid development of the aquaculture industry, requirements for testing involving fishes as indicators of environmental change, and the use of fishes as a replacement for mammals in biomedical, pharmacological and genetic research (DeTolla *et al.*, 1995; Fabacher & Little, 2000). The trend toward the use of fishes as a replacement for studies that would previously have used mammals as experimental subjects is not discouraged. However, it must also be recognized that fishes have the capacity to perceive noxious stimuli. Noxious stimuli are those stimuli that are damaging or potentially damaging to normal tissue (e.g., mechanical pressure, extremes of temperature and corrosive chemicals). Whether or not fishes have the capacity to experience any of the adverse states usually associated with pain in mammals is subject to a great deal of debate in the scientific literature (FAWC, 1996; FSBI, 2002; Rose, 2002; Braithwaite & Huntingford, 2004). Nonetheless, fishes are capable of behavioral,

physiological and hormonal responses to stressors (including noxious stimuli) which can be detrimental to their well-being. These CCAC guidelines both support the leadership role that Canadians play in fish research, and ensure that the welfare of fishes is carefully considered during the use of fishes for research, teaching and testing, recognizing that better welfare will result in better science.

3. Ethical Overview

Guideline 1:

Fishes used in research, teaching and testing must be treated with the respect accorded to other vertebrate species.

The CCAC's surveillance system for animals used in research, teaching and testing is based on the principles of humane science, i.e. the Three Rs of Russell and Burch (Russell & Burch, 1959) - Reduction, Replacement and Refinement. For the CCAC, these principles are laid out in its *policy statement on: ethics of animal investigation* (CCAC, 1989). The *ethics of animal investigation* applies to all species covered by the CCAC system, i.e. all vertebrates and cephalopods.

In addition, the CCAC system takes a "moral stewardship" approach to the use of animals in science as explained in the CCAC Experimental Animal User Training Core Topics - Module 2, Ethics in Animal Experimentation (http://www.ccac.ca/en/CCAC_Programs/ETCC/Module02/toc.html).

The first guideline statement in the CCAC *guidelines on: institutional animal user training* (CCAC, 1999a) states, "Institutions must strive through their training programs to sustain an institutional culture of respect for animal life".

3.1 Principles of the Three Rs

According to the CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989), it is the responsibility of the local animal care committee (ACC) to ensure that fishes are used only if the investigator's best efforts to find a non-animal model have failed.

As for any other species covered by the CCAC system, investigators using fishes are required to use the most humane methods on the smallest

number of animals necessary to obtain valid information. This requires the use of a sound research strategy, including: identification of key experiments that determine whether a particular line of enquiry is worth pursuing; use of pilot studies; staging of *in vitro* to *in vivo* experiments where possible; and implementation of staged increase in test stimuli where possible (Balls *et al.*, 1995). The numbers and species of animals required depend on the questions to be explored. Field studies, aquaculture studies and laboratory studies require different statistical designs; field studies and aquaculture production typically require the use of larger numbers of animals. The life stage of the fishes used in each study will also affect the numbers of animals needed. Studies of early life stages typically require large numbers of individuals. In all cases, studies should be designed to use the fewest animals necessary. Heffner *et al.* (1996) and Festing *et al.* (2002) provide discussions on the appropriate treatment of samples and experimental units. Investigators are encouraged to consult with a statistician to develop study designs that have the appropriate statistical power to accomplish the research objectives (Nickum *et al.*, 2004).

The CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989) also requires adherence to the following principles:

- animals must be maintained in a manner that provides for their optimal health and well-being, consistent with the demands imposed by the experimental protocol;
- animals must not be subjected to pain and/or distress that is avoidable and that is not required by the nature of the relevant protocol;
- expert opinion must attest to the potential value of studies with all animals, including fishes (e.g., scientific merit for research, see CCAC *policy statement on: the importance of independent scientific merit of animal based research projects* [CCAC, 2000a]; pedagogical value for teaching; and the appropriateness of the method to provide data for testing according to current regulatory requirements);
- if pain or distress is a justified component of