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The Effect of Temperature Differences on The Gonad Maturity Levels and Embryogenesis of Vaname Shrimp Broodstock (*Litopenaeus vannamei*)

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Abstract

Vaname shrimp cultivation in Indonesia is currently the mainstay of the aquaculture sector, in the 2012 - 2018 period, the contribution of the export value of vaname shrimp to the export value of Indonesian fisheries averaged 36.27%. The production of cultivated shrimp nationally has increased rapidly in the last 5 years with an average annual increase of 10.38%. To increase the production of vaname shrimp, it is necessary to observe the factors that can accelerate the level of gonadal maturity and embryogenesis in vaname shrimp. One of the environmental factors that affect the level of gonadal maturity and embryogenesis is temperature, temperature affects the sinus glands to accelerate the development of gonadal maturity levels, temperature also affects the process of embryo development, at high temperatures the shrimp metabolism rate increases and embryo development occurs more quickly. This research is experimental by using a Completely Randomized Design (CRD). This studyused four treatments, namely four temperature treatments, including 28°C, 30°C, 32°C, and 34°C. The results of the study were analyzed using ANOVA (Analysis of Variance) and continued with Duncan's Multiple Range Test. The results of this study indicate that temperature differences affect the level of gonadal maturity level (GML)and embryogenesis in the spawning process of vaname shrimp (Litopenaeus vannamei) broodstock ($p<0.05$). The treatment that had a major influence on the level of gonadal maturity level (GML) was the 34°C treatment with 8 days and the fastest embryogenesis treatment at 34°C with 387.3 minutes.

Keywords: *Litopenaeus vannamei*, gonad maturity level, embryogenesis

Introduction

Vannamei shrimp is a native species of Pacific waters that can be found on the west coast of Mexico to Peru. This shrimp was introduced for cultivation in Asia in 1996 in Taiwan by importing vannamei shrimp parent candidates from Hawaii. Furthermore, these efforts returned to China, Myanmar, Indonesia, and several countries in Southeast Asia. Vannamei shrimp has several advantages compared to other species, namely, the growth rate reaches 1-1.5 g/week, it can be cultivated with a high stocking density of around 80-500 individuals/m2, vannamei shrimp are also tolerant of salinity 0.5-45 ppm, as well as the harvest size, is uniform and the number of undersize is lower. (KKP, 2021).

Observation of factors that can accelerate the level of gonadal maturity and embryogenesis in vannamei shrimp is still rarely carried out, especially regarding environmental factors, one of the environmental factors that affect the level of gonadal maturity and embryogenesis is temperature, temperature affects the sinus glands to accelerate the development of gonadal maturity levels. , temperature also affects the process of embryo development, at high temperatures the shrimp metabolic rate increases and embryo development occurs more quickly. One alternative way to increase vannamei shrimp production is to carry out temperature treatment to accelerate gonadal maturity and embryogenesis in vannamei shrimp.

Based on the description above, the researchers conducted a study entitled "The Effect of Temperature Differences on Gonad Maturity Levels and Embryogenesis in Vaname Shrimp (*Litopenaeus vannamei*) Broodstock".

Methods

The method used in this research is experimental or trial. The research design used was a completely randomized design (CRD) using 4 treatments with 3 replications for each treatment. The treatment given was a temperature difference design in observing the level of gonadal maturity and embryogenesis of vaname shrimp (*Litopenaeus vannamei*).

Results and Discussion

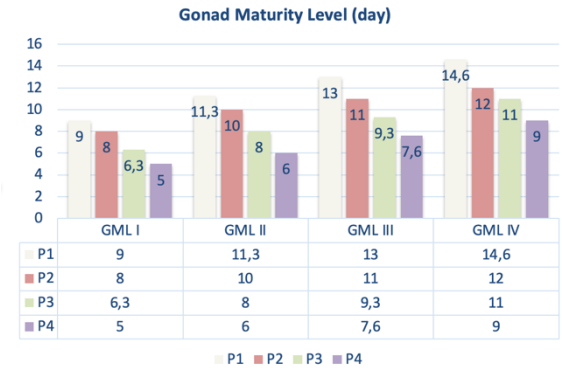

Figure 1.

Based on figure 1, P3 and P4 provide a faster response time needed to achieve GML I and II compared to P1 and P2. To reach GML I at temperature P4 it takes 5 days, at P3 it takes 6.3 days, at P2 it takes 8 days, while at P1 it takes 9 days. To achieve GML II at P4 it takes 6 days, at P3 it takes 8 days, at P2 it takes 10 days, while at temperature P1 it takes 11.3 days. To achieve GML III at P4 it takes 7.6 days, at P3 it takes 9.3 days, at P2 it takes 11 days, while at P1 it takes 13 days. To achieve GML IV, P4 takes 9 days, P3 takes 11 days, P2 takes 12 days, and P1 takes 14.6 days. This time difference gets smaller as we reach GML IV. This shows that the higher the treatment temperature, the faster the maturity level of the gonads. This is following the statement of Saputra (2013) which states that the maturity of the gonads of shrimp is influenced by 2 factors, namely internal factors and external factors. Internal factors include species and age. While external factors that influence include: temperature, food availability, water flow, and high rainfall. Stimulation from outside can affect the central nervous system which can inhibit organ X from producing Gonad Inhibiting Hormone (GIH). (Tiu and Chan, 2007).

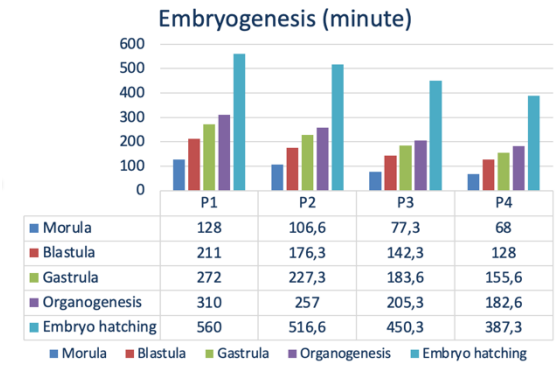

Figure 2.

Figure 2 shows that at the Morula stage, the fastest treatment was P4 with a time of 68 minutes, then P3 with a time of 77.3 minutes, P2 with a time of 106.7 minutes, and the slowest was P1 with a time of 128 minutes. At the Blastula stage, the treatment with the fastest time to reach this stage was P4 with 128 minutes and P1 showed the slowest time with 211 minutes. P2 takes 176.3 minutes, while P3 takes 142.3 minutes. At the Gastrula stage, P4 was also superior compared to other treatments with a time of 155.6 minutes, followed by P3 with a time of 183.6 minutes, P2 took 227.3 minutes and P1 took the longest with 272 minutes. At the stage of organogenesis, P4 was still the fastest to reach this stage with a time of 182.6 minutes, then followed by P3 with 205.3 minutes, then P2 took 257 minutes, and finally P1 with 310 minutes. At the last stage of embryo hatching, P4 was still the fastest to reach this stage with a time of 387.3 minutes, followed by P3 with a time of 450.3. P2 takes 516.6 minutes and the last one is P1 with a record time of 560 minutes. According to Andrianto et al. (2013), stated that temperature changes greatly affect the development of the embryo, because it affects the speed of metabolism, metabolism is a biochemical process that occurs in the body which is strongly influenced by temperature.

Water Quality Parameters	Temperature 28°C (P1)	Temperature 30°C (P2)	Temperature 32°C (P3)	Temperature 34°C (P4)	SNI 8037.1:2014
DO (mg/L)	7,84 – 8,14	7,49 – 8,04	6,08 – 6,54	5,68 – 6,12	>4,0
Temperature (°C)	28,1 – 28,9	30,1 – 30,7	32 – 35,6	34 – 34,6	28 – 33
pH	7 – 8,2	6,2 – 8,1	6,3 – 7,3	5,8 – 7,1	7,5 – 8,5
Salinity (ppt)	32-33	32-34	32-34	32-34	25 – 30

Table 1

The results of measuring water quality parameters at 28°C, 30°C, 32°C, and 34°C showed differences in temperature, dissolved oxygen (DO), pH, and salinity. Dissolved oxygen at 34°C showed a lower value of 5.68-6.12 mg/L, while at 28°C, 30°C and 34°C dissolved oxygen reached 7.84-8.14 mg/dL, 7.49-8.04 mg/L, and 6.08-6.54 mg/L. The temperatures in these four studies ranged from smallest to lowest, namely at a temperature of 28.1-34.6°C with different values for each treatment. P1 showed the lowest temperature, namely 28.1-28.9°C, while the temperatures of other treatments showed higher, including P2 30.1–30.7°C, P3 32–34.6°C, and P4 34 –34.6°C. pH at 34°C temperature treatment showed the lowest value of 5.8–7.1 while at 28°C, 30°C and 34°C the pH

reached 7–8.2.6–8.1, and 6, 3–7,3. Salinity at 28°C showed the lowest value, namely 32–33 ppt, while other treatments at 28°C, 30°C, and 34°C showed the same salinity, namely 32–34 ppt.

The temperature range that is said to be good for the life of the vaname shrimp brood according to SNI 8037.1: 2014 is 28°-33°C. The results of pH measurements during maintenance ranged from 5.8-8.2 which is not following SNI 8037.1:2014. This can occur due to changes in pH values that vary depending on seawater temperature, dissolved oxygen concentrations, and the presence of anions and cations (Kumar et al., 2012). While the results of measuring salinity in the holding water of vaname shrimp broodstock are around a value of 32-34 ppt where this value is not following SNI 8037.1: 2014, especially at a salinity of 34 ppt.

Supono (2015) states that a high salinity value is caused by a high rate of water evaporation (evaporation of water) due to high temperatures coupled with a low level of mixing of new water, besides that it can also adversely affect shrimp osmoregulation. Vaname shrimp can live in the salinity range (0-31) ppt, but still grow well at (15-25) ppt and optimally at salinity (25-30) ppt (Hirono, 1992).

Conclusion

The difference in temperature affects the Gonad Maturity Level (GML) of vaname broodstock (*Litopenaeus vannamei*) ($p<0,05$). The treatment that has a major influence on the level of gonadal maturity (GML) is P4 (34°C) treatment within 9 days. The treatment that had the most direct effect on the process of embryogenesis was P4 (34°C) with a time of 387.3 minutes.

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Telah melakukan penelitian yang dipublikasi pada bulan November tahun 2022 dengan judul sebagai berikut:

The Effect of Temperature Differences on The Gonad Maturity Levels and Embryogenesis of Vaname Shrimp Broodstock (Litopenaeus vannamei)

Adapun penelitian ini sudah mengacu pada prosedur pertimbangan etik dari:

1. *American Fisheries Society* (AFS, 2014) yang berjudul *Guideline for the Use of Fishes in Research* yang menyebutkan bahwa: penelitian dalam kondisi laboratorium baru mengatur tentang hewan percobaan berupa ikan hidup, untuk hewan percobaan berupa udang (crustacean) tidak termasuk (hal 43 ; terlampir), dan
2. *Canadian Council on Animal Care* (CCAC, 2005) yang berjudul *Guideline on the Care and Use of Fish in Research, Teaching and Testing* yang menyebutkan bahwa: pedoman tersebut hanya digunakan untuk hewan uji berupa ikan (Kelas: Chondrichthyes, Agnatha, dan Osteichthyes) dan Avertebrata (Kelas: Cephalopoda) (hal 13,14 ; terlampir).

Sedangkan dalam penelitian tersebut menggunakan indukan udang vaname sebagai hewan percobaan. Sehingga penelitian tersebut tidak perlu dilakukan ***Uji Ethical Clearence.***

Demikian Surat Keterangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar** atas nama Dr. Endang Dewi Masithah, Ir., MP.

Surabaya, 27 April 2023

Mengetahui,
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Guidelines for the Use of Fishes in Research

Use of Fishes in Research Committee members:
J. A. Jenkins, Chair, H. L. Bart, Jr., J. D. Bowker, P. R. Bowser, J. R. MacMillan, J. G. Nickum, J. D. Rose, P. W. Sorensen, and G. W. Whitledge on behalf of the American Fisheries Society; J. W. Rachlin and B. E. Warkentine on behalf of the American Institute of Fishery Research Biologists; and H. L. Bart on behalf of the American Society of Ichthyologists and Herpetologists

American Fisheries Society
Bethesda, Maryland
2014

A suggested citation format for this book follows.

Use of Fishes in Research Committee (joint committee of the American Fisheries Society, the American Institute of Fishery Research Biologists, and the American Society of Ichthyologists and Herpetologists). 2014. Guidelines for the use of fishes in research. American Fisheries Society, Bethesda, Maryland.

Cover art: Close-up photograph of Brown Trout, *Salmo trutta*, from the South Fork of the Cache la Poudre River, Colorado, taken by James Rose in 2010.

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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

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.

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

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 relevent 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