UNIVERSITY OF OREGON

April 16, 2009

To Whom It May Concern:

The following application, # 09-04, was reviewed and approved by the University of Oregon Animal Care and Use Committee.

Title of Application:	Zebrafish International Resource Center
Name of Applicant:	Monte Westerfield
Name of Institution:	University of Oregon Institute of Neuroscience
Grant Number:	1 P40 RR012546, 1 R24 RR023998-01A1
ORSA Proposal Number:	10039, 10795, 11972,13608

Approved with the following modifications required:

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks. The Assurance number is A-3009-01.

Additional funding and personnel information related to this protocol:

AGENCY: NIH

PERSONNEL:

IACUC Member Designated
to Verify Acceptance

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
5218 University of Oregon, Eugene OR 97403-5218 T (541) 346-4958 F (541) 346-0588 www.uoregon.edu

Approval Date 4/16/09

1st Renewal Due Date _____

2nd Renewal Due Date _____

Expiration Date _____

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ANIMAL USE APPLICATION TO THE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE (IACUC) UNIVERSITY OF OREGON

ZEBRAFISH FORM

(Must be typewritten - Diskette available upon request in IACUC, OVSAC and some departmental offices)

Date Submitted April 6, 2009

I. TITLE, DATES, AND PERSONNEL

Title of Research Project **Zebrafish International Resource Center**

Project Dates 05/01/98 (new funding period began 05/01/08) to 04/30/13

Principal Investigator Monte Westerfield Title/Rank Professor / PI

Dept/Institute Institute Of Neuroscience Ext. 6-4607 Emergency Phone (541) 738-9499

Co-Investigator Zoltan Varga Title/Rank Co-PI/Director

Dept/Institute ZIRC Ext. 6-6099 Emergency Phone 541-915-7336

Other Personnel Katy Murray, Andrzej Nasiadka, April Freeman, Austin Bailey, David Lains, Carrie Barton, Carrie Carmichael, Ron Holland, Erin Quinn, Terra Hiebert, Renee Clark, Justin Bauer, Calvin Smith, Evan Williams, Andrew Van Gordon, Jen-Jen Hwang-Shum, Anwar Adi

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II. **PURPOSE** (Check if applicable):

☑ Research Project ☐ Pilot Project ☐ Teaching ☐ Student Special Project

III. **FUNDING (POTENTIAL AND AWARDED)**

A. **PROTOCOL STATUS:**

☑ New
☐ Amendment
☐ Annual Renewal PROJECT # 09-04

B. **FUNDING PROPOSAL TYPE:**

☐ New
☑ Continuation
☐ Renewal
☐ Revision

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Was this project originally funded or reviewed without the use of animals, or are there new significant changes involving animals which were not previously outlined in the grant proposal?

YES ☐
NO ☑

If yes, you will need to send a letter addressed to the program officer of the granting agency detailing the proposed significant changes involving the use of animals. The letter requires a counter-signature by the institution. The Office of Research Services & Administration (ORSA) will sign on behalf of Richard Linton, Vice President for Research. You may attach Section IX of the protocol application to satisfy funding agency requirements and IACUC policies and procedures. If you have any questions regarding the requirements for the letter, please contact ORSA, 346-5131, or OVSAC, 346-4957. Please attach a copy of the letter with the appropriate signatures to this application.

C. **EXTRAMURAL FUNDING**:

(When more than one funding source is solicited, a single IACUC animal use request may be submitted provided the species, number, and procedures are the same for each grant proposal application.)

Agency National Institutes of Health Grant # 1 P40 RRHD12546

Grant Title Zebrafish International Resource Center

Proposed Dates: 05/01/08 to 04/30/13

Agency National Institutes of Health Grant # 1 R24 RR023998-01A1

Grant Title Development of Germplasm Resources for Preservation of Aquatic Models

Proposed Dates: 09/01/08 to 05/31/11

D. **INTRAMURAL/NON-COMPETITIVE FUNDING** (See page 2 of instructions)

Funding Source N/A

Proposed Dates _____ to _____

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E. **COOPERATIVE RESEARCH**

Is this a cooperative research project (are there principal investigators from more than one institution involved)?

Yes ☑
No ☐

If yes, please see the UO's Policy to Address Cooperative Research which states that all Principal Investigators engaged in cooperative research with another institution must have approval by the UO's IACUC for all those projects which utilize vertebrate animals. Additionally, if the animals are housed at a cooperating facility, then there must be approval by the cooperating institution's IACUC.

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F. **PEER REVIEW OF UNSPONSORED RESEARCH**

(1. For teaching applications. 2. Other)

1. Departmental Curriculum Committee Review:

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N/A		
(Department)	(Committee Chair)	(Date Approved)

2. Other Peer Review:

I have reviewed the attached animal use application and find it to be scientifically valid and consistent with University of Oregon policy.

Signature (Authorized reviewer)	Name & Title (typed)
Signature (Authorized reviewer)	Name & Title (typed)

IV. ANIMAL REQUIREMENTS AND FACILITIES

NOTE: Approval does not necessarily guarantee that housing is available. It is the investigator's responsibility to make housing arrangements with the manager of the zebrafish facility.

Common Name Zebrafish **Source** ZIRC and various world wide labs

Strain various **# of embryos per year** ~8.1 x 10^6

# of adults per year* ~163,600

(*Adults used for experiments, not for maintaining the breeding colony.)

Housing Location Zebrafish International Resource Center.

Lab Room# ZIRC rms.103 and 118 **Will special housing be needed?** No

If yes, explain: _____ **Will animals be held more than 12 hours outside of OVSAC?** Yes **If yes, explain:**

All housing locations are outside of OVSAC; adults and larvae are kept within these locations.

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Will facilities outside of University of Oregon campus be used? No If yes, explain: N?A

Are animals wild or laboratory-bred? Laboratory bred

Is live feed required? Yes **If yes, explain:** Larval fish are fed live paramecium (protozoan). Fish of all life stages (larval-adult) are fed live *Artemia* nauplii (newly hatched brine shrimp).

V. WHAT IS THE OBJECTIVE OF THIS STUDY? HOW IS THIS STUDY RELEVANT TO HUMAN OR ANIMAL HEALTH, THE ADVANCEMENT OF KNOWLEDGE, OR THE GOOD OF SOCIETY? (In lay terms)

The last several years have witnessed an explosion in our understanding of vertebrate development, largely based on work from a few model genetic organisms. The embryonic zebrafish is the newest of these model organisms. Because the basic principles of body patterning appear similar during embryogenesis of all vertebrates, insights gained from work on embryonic zebrafish will have implications for human health and disease. Moreover, research on this organism meets the intent of the Animal Welfare Act because use of many higher vertebrates can now be replaced by use of this 'lower' vertebrate.

Genetic research on zebrafish was started at the University of Oregon and for many years Eugene was the only place it was performed. In the past decades however, international interest in this organism has grown tremendously (Balter, 1995); studies of the embryology and genetics of zebrafish have lead to a dramatic increase in the number of laboratories using this organism to study the basic mechanisms of vertebrate development. These laboratories have generated several hundred transgenic fish lines, identified over 20,000 genetic mutations, and due to the recent completion of the zebrafish genome sequencing project, plans are underway to produce a mutation in every gene of the zebrafish genome (up to ca. 35,000 genes). Most of the genetic stocks are distributed among more than 300 laboratories in 28 countries. To make room for new mutants, laboratories must discontinue some of their current stocks. Although mutations can be preserved as frozen sperm, not all laboratories are proficient with this technique. Thus, discontinued stocks may be permanently lost unless a central site serves as a repository to preserve and keep track of these stocks for future research.

The Zebrafish International Resource Center acquires, and maintains wild-type, transgenic, and mutant zebrafish stocks and makes them widely available to the international research community. Our specific objectives are:

1. To serve as a central repository for zebrafish genetic stocks and research materials. We will maintain healthy stocks of fish and frozen sperm of identified genotypes and make them widely available to the research community. We will continue to expand our collection by obtaining carriers of mutations from the research community and breed them to produce new generations. We will freeze and store sperm from all these lines. We will acquire the most widely used wild-type lines and maintain them in a manner that preserves their genetic diversity. We will receive and store antibodies, gene probes and markers used to identify and analyze wild-type and mutant stocks. Upon request, we will ship fish and materials to research

laboratories throughout the world. We will provide online information about the stocks, materials, ordering procedures and methods http://zebrafish.org/zirc/home/guide.php
in collaboration with ZFIN, the zebrafish model organism database.

2. To provide consultation and pathology services. We will provide diagnostic services and health status testing for laboratory zebrafish. We will use histopathology, bacteriology, necroscopy, and virology to analyze specific or suspected disease problems. We will provide routine sentinel or quality control testing of zebrafish from healthy laboratory colonies for early detection of problems and to fulfill institutional animal care and use committee, IACUC, health status monitoring requirements. We will continue to develop methods to detect and control disease in laboratory colonies. We will continue to investigate the transmission and pathology of the microsporidian parasite, *Pseudoloma neurophilia,* and *Mycobacterium chelonae.* We will maintain and update our online manual for the prevention, diagnosis and treatment of diseases affecting zebrafish: http://zebrafish.org/zirc/health/diseaseManual.php

3. To develop improved zebrafish husbandry methods. We will establish standards and procedures for generating healthier and more vigorous colonies. We will study the influence of stress, diet, water conditions, housing, and husbandry on larval and juvenile growth, adult fecundity, reproductive longevity and disease. We will develop and improve standardized methods for cryopreservation and reconstitution of genetic lines.

VI. DOES THIS STUDY UNNECESSARILY DUPLICATE PREVIOUS EXPERIMENTS? IF SO, EXPLAIN.

We confidently assert that the studies that the Resource Center will support do not unnecessarily duplicate previous work.

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CHECKLIST. (Please give details in Section IX).

VII. TYPE OF PROJECT

(If necessary, please consult OVSAC for further information concerning pain categories. This section is only a checklist.)

PAIN CATEGORY (Indicate species and number of animals in each pain category):

C 439,440adult zebrafish and 24.35 x 10^6embryos

Procedures that are considered to produce minimal, transient, or no pain or distress when performed by competent individuals (e.g. all zebrafish embryos**, euthanasia).

D 51,360 adult zebrafish

Procedures or tests involving the administration of appropriate anesthetic, analgesic, or tranquilizer drugs to avoid pain or distress (e.g., fin clips, MS222/tricaine on adults from which sperm and eggs are squeezed, ENU).

E* N/A

Procedures or tests that, for scientific validity, are performed involving pain or distress without administration of appropriate anesthetic, analgesic, or tranquilizer drugs.

* **NOTE:** when a protocol falls into the "E" category, the investigator must attach a written justification for the procedure and may be requested to attend an IACUC meeting to discuss the proposed research.

** **In practice, tricaine anesthesia is sometimes used to facilitate capture and handling of the fish at any stage after the embryos become motile even though the procedures produce no or minimal discomfort. Even invasive procedures done with embryos could not produce discomfort because the neural centers mediating pain sensation are still undeveloped.**

PROCEDURE

☐ Blood Collection ☑ Surgical ☐ Non-Surgical ☐ Behavioral ☐ Field Study

☑ Other (Describe): Care and maintenance of adults; breeding and obtaining gametes and embryos (including parthenogenetic embryos); raising larvae, cryogenic preservation of sperm; strain record-keeping; fin clips, quarantine and other procedures relating to disease control and euthanasia

TYPE OF STUDY

☑ Terminal (Acute): Animal never awakens from initial procedure.

☑ Survival (Chronic): Animal awakens and survives for _____hours/days after initial procedure.

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SPECIAL CONSIDERATIONS:
(Check all that applies and explain all checked in Section IX)

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☐ Multiple surgeries
☐ Restraint device(s)
☐ Neuromuscular blocking agents
☐ Complete Freund's Adjuvant (If checked, include signed copy of the U of O Adjuvant Policy)
☑ Breeding Colony (If checked, include the SOP for care and breeding)
☐ Food or Water Deprivation

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VIII. ANIMAL EXPERIMENTATION INVOLVING HAZARDOUS AGENTS

Are any hazardous agents including infectious agents, biohazards, carcinogens (ENU for mutagenizing), toxic chemicals, or radioisotopes, gamma rays for mutagenizing used on live animals for this study?

☐ Yes
☑ No

If hazardous agents are being used, attach a use authorization from the appropriate committee or office.

Authorized by:

1. Biosafety Committee (Infectious agents and biohazards)? ☐ Yes ☑ No
2. Environmental Health & Safety Office (Carcinogens and toxic chemicals)? ☐ Yes ☑ No
3. Radiation Committee (Radioisotopes)? ☐ Yes ☑ No

NOTE: Since the use of animals in experimentation involving hazardous agents requires special consideration, the procedures and the facilities to be used must be reviewed by both the Office of Environmental Health and Safety and the IACUC. Formal safety programs should be established to assess the hazards, to determine the safeguards needed for their control, and to ensure that the staff is competent, and that the facilities are adequate for the safe conduct of the research (PHS *Guide*).

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IX. PLEASE PROVIDE DETAILED INFORMATION FOR THIS SECTION ON A SEPARATE SHEET - **Instructions** (for Detailed Information section XI - see following pages)

This application form has been reformatted in order to accommodate the Vertebrate Animal Section of the Research Plan of the Public Health Service Grant Application form, PHS 398. Items 1-5 in the bold print are quoted directly from the PHS Application Packet. The light print is to serve as a guide (check sheet) in preparing your response to meet funding agency and IACUC requirements. This format is applicable for all animal use protocols, even when the funding source is other than PHS. **If PHS is the funding source, please answer the following questions and attach a copy of the Vertebrate Animal Section of the Research Plan. For funding other than PHS, please answer the following questions and attach a copy of all relevant portions of the grant application pertaining to animal care and use.**

1. **Provide a detailed description of the proposed use of animals in the work previously outlined in the experimental design and methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.**
   Experimental/Non Surgical Study: Identify procedure and duration of study.
   Behavioral Study: Describe any conditioning, deprivation, or stimulation that might be involved.
   Field Study: For capture or any invasive procedure
   For Surgical, Blood & Tissue Collection, Address:
   - Drugs and/or antigens used — Quantity
   - Route of administration — Frequency
   - Injection sites — Pain associated with procedures

   For Surgical Procedure, Address:
   - Pre-operative care — < Methods to prevent dehydration/hypothermia
   - Surgical procedure — < Anticipated duration of surgery
   - Multiple surgeries — < Anticipated duration of surgeries/type
   - Use of paralyzing drugs — < Anticipated duration of study/endpoint/pain
   - Post-operative care — < Anticipated nursing care medication & duration
   -

2. **Justify the use of animals, the choice of species, and the numbers used. If the animals are in short supply, costly, or to be used in large numbers, provide additional rationale for their selection and their numbers.**

3. **Provide information on the veterinary care of the animals involved.**
   NOTE: It is not necessary to complete this section for the IACUC. It is only necessary to state that veterinary service is being provided by the Office of Veterinary Services & Animal Care as described in routine facility standard operating procedures or PHS-approved assurance statements.

4. **Describe procedures of ensuring that discomfort, distress, pain and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices where appropriate to minimize discomfort, distress, pain and injury.** Address:
   - **Analgesic/anesthetic/tranquilizing drugs**
     - Dose — o Frequency
     - Route of administration — o Criteria to assess pain/discomfort
   - **Describe use of comfortable restraining devices**
     - Dimensions and/or type
     - Duration of confinement (continual observation required)
   - **Describe any other animal manipulations that may produce pain, discomfort, or anxiety not mentioned previously**
   - **Describe any physical or psychological impairment of the animal resulting from experimental manipulation (e.g. blindness, loss of motor abilities)**
   - **Describe the methods used to assess ~~adequate levels of~~ anesthesia**
   - **Describe indices used to help assess possible signs of pain, distress or discomfort**

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5. **Describe any euthanasia method to be used and the reasons for its selection. State whether this method is consistent with the recommendations on the panel of euthanasia of the American Veterinary Medical Association available in the OVSAC library and the *Researcher's Handbook*. If not, present a justification for not following the recommendations.**
   A. For chemical or gas euthanasia, please include the agent, dose and route.
   B. For physical euthanasia, please indicate the specific method:

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IX. Detailed Information

If PHS is the funding source, please answer the following questions and attach a copy of the Vertebrate Animal Section of the Research Plan. For funding other than PHS, please answer the following questions and attach a copy of all relevant portions of the grant application pertaining to animal care and use.

INTRODUCTION: Standard Description of the Proposed Use of Animals

Species: Zebrafish, *Danio rerio*
Sex: both females and males
Ages: all ages
Number: Approximately **490800 adults and 24.35 x 10^6 embryos**

The zebrafish group has prepared its own detailed user manual that describes standard procedures. This was done because of the special requirements of zebrafish, and how they were used, in contrast with the use of other vertebrates at the University of Oregon (particularly birds and mammals), that are covered by OVSAC's Standard Operating Procedures Document. The zebrafish standard operating procedure manual is *The Zebrafish Book* (ed. 5, 2007). The manual has been approved by the IACUC and is currently included within the IACUC packet.

The Zebrafish Standard Operating Procedures covered by this application do cover all procedures, including invasive ones, carried out with embryos, either done within the facility (e.g. DNA injection into early embryonic cells) or in the user laboratories (e.g. cell labeling, microsurgery, laser microablation, cell transplantation, donors for cell & tissue culture). In addition, all usual facility and husbandry operations are included: care and maintenance of adults, breeding and obtaining gametes and embryos (including parthenogenetic embryos), raising larvae, cryogenic preservation of sperm, fin clips, mutagenesis, strain record keeping, sending fish to and receiving fish from other laboratories, quarantine and other procedures relating to disease control, and euthanasia.

1a. **Briefly summarize the methods to be used in achieving the objectives of your proposal. Please emphasize any procedures not covered by the *The Zebrafish Book* (please give a brief description in the space provided below).**

STANDARD METHODS LISTED IN THE ZEBRAFISH BOOK

Chapter 1 - GENERAL METHODS FOR ZEBRAFISH CARE and Husbandry

1. Availability and simple care
2. Food
3. Water
4. Quarantine Room Procedures
5. Cleaning Tanks
6. Shipping Fish
7. Keeping Track Of Stocks
8. Zebrafish Standard Protocol Form

Chapter 2 - BREEDING

1. Simple Method For Steady, Low-Level Embryo Production
2. Method For Maximal Embryo Production
3. Embryo Collection
4. Simple Schedule For Breeding Zebrafish
5. Zebrafish Breeding Schedule For Maximal Embryo Production
6. Pair-Wise Breeding Of Individual Or Natural Cross Fish
7. Embryo Production By In Vitro Fertilization
8. Overview of in vitro fertilization
   a. General Procedures and Overview
   b. Squeezing Males
   c. Squeezing Females
   d. In Vitro Fertilization

Chapter 3 - EMBRYONIC AND LARVAL CULTURE

1. A Simple Method for Raising Babies
2. Raising Larvae in a Nursery

3. Simple, Quick Methods For Raising Paramecia
4. A Semi-Sterile Method For Raising Paramecia
5. Device for harvesting paramecia
6. Growing Paramecia Under Sterile Conditions
7. Paramecia Culture Medium
8. Purification of Paramecia
9. A Simple Method for Raising Larvae
10. Developmental Staging Series

Chapter 4 - MICROSCOPIC OBSERVATIONS

1. Removing Embryos From Their Chorions
2. Viewing Chambers
3. Agar Mounting
4. Methyl Cellulose Mounting

Chapter 7 - GENETIC METHODS

1. Conventions For Naming Zebrafish Genes
2. Suggested Guidelines For Maintaining Mutant Stocks
3. Overview Of Methods For Parthenogenesis
4. Production Of Haploid Embryos
5. Production Of Homozygous Diploid Embryos
6. Heat shock
7. Early pressure
8. Delayed In Vitro Fertilization Using Coho Salmon Ovarian Fluid
9. Freezing Sperm
10. Thawing And Using Frozen Sperm For In Vitro Fertilization
11. Fin Amputations
    a. Testosterone Treatment To Produce Males
    b. Genetic Strains

Chapter 9 - MOLECULAR METHODS

1. Preparation Of Genomic DNA
2. Rapid Isolation Of RNA From Zebrafish
3. Total Nucleic Acid Extraction Procedure For Zebrafish Embryos

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NON-STANDARD METHODS:

1) Euthanasia

See sections 5.a/5.b as already approved by the IACUC.

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2) Disease research

a) An extensive health surveillance program includes pre- *and* post-filtration sentinel fish in the main facility, daily health monitoring, and an in-house diagnostic pathology service. These fish receive standard husbandry and care as described in the Zebrafish Book. Sentinel fish that have been in the sentinel tanks for three and six months are euthanized quarterly and subjected to post-mortem diagnostics (histopathology). The results of diagnostic tests are available on-line http://zebrafish.org/zirc/documents/health_report.php

b) In the wild-type strains housed at ZIRC, we investigate prevalence of and predisposition for two types of pathogens: Mycobacteria and Microsporidia. This means that we house fish under normal conditions in standard tanks and collect data to identify overall prevalence of a pathogen in the facility, or within a particular wild-type strain. We are determining the effects of tank size and methods of cleaning on the prevalence of infections. These experiments may involve exposure to biofilms from other tanks. All other procedures involve standard techniques and procedures described in the Zebrafish Book.

3) Toxicity testing.

When new types of aquaria, or aquaria components (in particular plastics) become available through the manufacturer of our rack systems, we typically test such components first to ensure that no toxic compounds leach from them into the water system. To this end, limited numbers of embryos, larvae, and adults, are housed in aquaria together with such parts. We observe whether the animals are affected in their health and behavior in any way, whether fish fecundity is affected, and whether the offspring suffer from any conditions. We also subject these fish to diagnostic histopathology testing

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to search for potential tumors, organ malformations etc that could stem from toxic compounds emanating from the new tank components.

2a. **Standard justification for the use of animals and choice of species:**
Zebrafish have become widely accepted as a particularly useful organism to analyze how vertebrate development is regulated at the cellular, genetic, and molecular levels. Zebrafish offer several advantages:

(1) They are easy to maintain in large numbers and readily reproduce under laboratory conditions;
(2) Adults can be subjected to mutagenesis and mutations can be screened in the first generation by analyzing haploid embryos (not done at ZIRC);
(3) Embryos have few cells compared to other vertebrates, thus making it a "simpler" model for more complex vertebrates, including human;
(4) Embryos are optically transparent, develop very rapidly, and externally (not inside the mother or an eggshell) so that tissue development can be readily observed;
(5) Direct access to the developing embryos makes it possible to introduce foreign genetic material and to perform cell labeling and other experimental manipulations (not performed at ZIRC); and
(6) Zebrafish are small, so that large numbers, required for genetics, can be maintained and studied.

2b. **Are there any other justifications for this project not outlined above?** No
If yes, please list.

2c. **Justify the number of animals proposed:**
The numbers in this section are *projections*. They are in part based on actual numbers reported to the External Advisory Board in 2007 (a recent, representative year). However, we also considered an estimated increase in numbers, consistent with the average increase rates in 2006 – 2008 for imports, requests/shipping, and husbandry turnover (generations of fish, lines of fish). For actual numbers, refer to our Annual Advisory Board reports.

a) Currently, we house 1148 distinct zebrafish lines (most are cryopreserved sperm) in the facility, and keep the most requested ones, approximately 400 lines, as live stock. ZIRC can maintain up to 500 genetically distinct, live zebrafish lines. We need an average of 150 fish to maintain each line. This requires about 500 x 150 = 75,000 adults. Typically, new generations are produced every 6 months, so approximately **150,000 adult fish** will be maintained in the facility each year **[450,000 adults total]**.

Mutant lines are maintained by crossing mutant carriers with wild-types and growing up the offspring. Only 2 out of 4 of these offspring will inherit the mutant chromosome. To identify carriers from this new generation, siblings are crossed to each other and on average 4 crosses will need to be done to identify a pair of carriers. On average, approximately 200 embryos are produced by each cross. This process will be repeated twice a year to have young, healthy breeders available at all times. To identify 10 carriers per line, we will generate:

10 carriers x 4 crosses x 200 embryos x 500 lines x 2 times per year $= 8 \times 10^6$ **embryos [24 x 10^6 embryos total]**.

The Resource Center provides adult zebrafish and embryos to research laboratories throughout the world. In 2007, the Resource Center supplied 8,600 adult zebrafish and 103,000 embryos to the research community. We expect the customer demand for zebrafish embryos and adults to increase over the coming three years. The estimated number of adults we will ship over the following three years is 30,000 fish. The estimated number of embryos that we will ship during the coming three years is 350,000 embryos.

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For the coming 3 years, we estimate to handle a total of
[480,000 adults (450,000 + 30,000) and **24.35 x 10^6 embryos** (24 x 10^6 + 0.35 x 10^6)].

Per year, approximately, 7,000 of these are anesthetized and fin-clipped for genotyping by polymerase chain reaction (PCR) [21,000 adults total]. In addition, routine cryopreservation to freeze sperm from lines at the ZIRC accounts for approximately 3000 males need to be anesthetized. In addition, when reviving frozen lines by in vitro fertilization (IVF), we need to anesthetize females, squeeze them to produce eggs, which we fertilize with thawed sperm. We estimate that for cryopreservation test-thaws (quality control) and for shipping to customers from thawed sperm, we will use ca. 5500 females. All these are a subset of the 450,000 adults and do not require additional breeding. However, they also belong into pain category D (see page 6, and Table below).

b) Experiments on optimizing husbandry conditions will be performed on wild-type (AB) shipping stock and will not require additional fish (Pain category C).

c) Experiments on fish cryopreservation with our collaborators at LSU will require an additional 1600 adults to be generated per year. For this study, ZIRC breeds, raises and ships fish to LSU. The cryopreservation research at LSU is covered by the approved IACUC protocol there, the research at ZIRC is covered by the standard operations at ZIRC (Zebrafish, Book) detailed **[4800 total fish].**

d) Experiments on the transmission and pathology of *Pseudoloma neurophilia* and *Mycobacterium cholonae* are expected to utilize additional 1500 adult fish per year **[4500 total fish].**

e) Toxicity studies will utilize an estimated 500 larvae that are raised to adulthood each year. Therefore, we consider these adult fish and will breed a total of **[1500 total fish].**

Overview: Projected Number of Animals Used iver 3 Years and Pain Category

Project	Adults Total	Adults, Category C	Adults, Category D	Embryos (Cat. C)
a) Routine Maintenance	480 000	Husbandry studies & Routine breeding: 433 500	Cryopreservation: 9 000 IVF: 16 500 Fin clipping: 21 000	Routine Breeding: 24 x 10^6 Shipping: 3.5 x 10^5
c) Cryopreservation Research w/ LSU	4800	-	4 800	-
d) Transmission & pathology studies	4500	4 440	60	-
e) Toxicity studies	1500	1500	-	-
Total per year	**163 600**	**146 480**	**17 120**	**8.11 x 10^6**
Total (3 years) per Pain Category	**490 800**	**439 440**	**51 360**	**24.35 x 106**

3. **Veterinary Care:**

Veterinary care is provided by the Resource Center staff; Dr. Katy Murray, ZIRC veterinarian. She consults with OVSAC and Dr. Alexander Ojerio as required. Dr. Michael Kent, from OSU consults with Dr. Murray as a fish pathologist on external ZIRC Pathology Service cases.

4a. **Standard Procedures for Alleviation of Pain, Discomfort, Distress, and Injury:**

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Most of the procedures on embryos will be done at very early developmental stages before the nervous system has matured. Indeed, neural crest cells are the *source* of sensory neurons that ultimately develop in these organisms. We feel, therefore, that without the structures necessary to detect pain, embryos at this stage are unlikely to be susceptible to painful stimuli. On the other hand, the developing muscle cells in the embryos twitch spontaneously causing the embryos to move. To prevent such movements, which make observations of cells more difficult, embryos older than 17 hours will be anesthetized in Tricaine, also called MS 222, added to the water. Tricaine is the best anesthesia available for lower (aquatic "cold-blooded") vertebrates. The dosage is age dependent. Anesthesia is administered by immersing the animal in the anesthetic to facilitate handling of the fish, e.g. during procedures to obtain gametes from adults which involves handling of the fish but produces minimal discomfort even if the fish were alert. There is no permanent impairment.

4b. **Which standard procedures outlined above or any others not mentioned will be utilized to ensure minimization of pain, discomfort, distress and injury?**

The only non-standard situation at the Resource Center will involve disease, or toxicity studies. Fish may be exposed to agents in one of three ways, by immersion (category C), by feeding (category C) or by injection of anesthetized fish in order to make the fish easier to handle (category D, performed only at LSU). Cohorts of potentially infected fish will be maintained through the study period until they are sacrificed to assess the results of the experiment. Individual fish that are displaying obvious signs of disease prior to that time will be sacrificed in order to minimize their experience of pain and to preserve the usefulness to the study (category C).

5a. **Standard method of euthanasia:**
Standard methods of euthanasia include:

1) For all developmental stages of zebrafish, hypothermal shock by immersion in ice water following IACUC approved SOP on Zebrafish Euthanasia.

2) For adults, immobilization by submersion in ice water immediately followed by cranial concussion and decapitation via an in-sink garbage disposal.
3) For adults, overdose of tricaine methanesulfonate (MS-222, 200-300mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
4) Anesthesia with tricaine methanesulfonate (MS-222, 168mg/l) followed by rapid freezing in liquid nitrogen.

5b. **Which standard method or other will be utilized?**
Only method 1) is used at ZIRC. Carcasses are disposed of by in-sink garbage disposal.

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X. PERSONNEL[1]

Please complete one of these pages for each P.I., research assistant, student, postdoc, and primary lab employee who will have direct animal and/or tissue contact in this study (including antibody use and antigen preparation for antibody production).

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PERSONNEL QUALIFICATIONS AND TRAINING

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A. P.I. QUALIFICATIONS AND TRAINING

NAME Monte Westerfield — Campus Phone 6-4607
Position Professor of Biology — Institute/Department Institute of Neuroscience
Credentials/Experience 25 years experience with zebrafish, trained by George Sreisinger
Work Location Room 337 — Emergency Phone 343-5637
E-Mail Address monte@uoneuro.uoregon.edu

In accordance with federal regulations, please provide the following information. Be advised that the P.I. must assure that all persons participating have demonstrated competence required to perform techniques listed below.

B. LAB PERSONNEL QUALIFICATIONS AND TRAINING

I have read the protocol and understand my responsibilities outlined therein. I have also read the University of Oregon's Animal Care & Use Training Handbook.

Name	Credentials/Experience	Personnel Signature	Trained By	Training Required (Y/N)	OHP Review Date
Zoltan Varga, Ph.D	4 years post-doc in Monte Westerfield lab, 4.5 years experience with zebrafish as Assistant Professor in Freibrug Germany, 5 years as director of ZIRC		Monte Westerfield, ZIRC Staff	No	10/2004
Katy Murray, DVM, Ph.D.	6 years licensed veterinarian 4 years experience at UO Aquavet I & II training 1.5 years experience as ZIRC veterinarian		Zebrafish Facility Staff	No	8/2006
April Freeman (Mazanec)	3 years experience with UO Zebrafish Facility, 10 years experience with the ZIRC, 8 years assisting ZF course in Woods Hole		Zebrafish Facility Staff	No	3/2001
Erin Quinn	5 years experience with ZIRC		ZIRC Staff	No	Pending
Andrzej Nasiadka Ph.D.	3 years experience at the University of Freiburg, 5 years experience at ZIRC		Driever Lab, ZIRC Staff	No	10/2004
Austin Bailey	5 years experience with UO Zebrafish Facility, 9 years experience with ZIRC		Zebrafish Facility Staff, ZIRC staff	No	5/2000

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David Lains	8 years experience with ZIRC, attended aquaculture conferences		Zebrafish Facility Staff, ZIRC staff	No	1/2001
Carrie Barton	4 years experience with UO Zebrafish Facility, 8 years experience with ZIRC		Zebrafish Facility Staff, ZIRC staff	No	3/2002
Carrie Carmichael	2 years experience with UO Zebrafish Facility, 7 years experience with ZIRC		Zebrafish Facility Staff, ZIRC staff	No	1/2002
Terra Hiebert	4 years experience with the ZIRC		Zebrafish Facility Staff, ZIRC staff	No	6/2005
Jen-Jen Hwang-Shum, Ph.D	4 years experience with ZIRC		Zebrafish Facility Staff, ZIRC staff	No	10/2005
Renee Clark	4 years experience with ZIRC		Zebrafish Facility Staff, ZIRC staff	No	4/2005
Ron Holland	6 years experience with ZIRC		ZIRC Staff	No	3/2004
Anwar Adi	11 months experience at ZIRC		ZIRC Staff	No	Pending
Andrew Van Gordon	2.5 years experience at ZIRC as student employee, 6 months experience as a ZIRC research assistant		Zebrafish Facility Staff, ZIRC staff	No	8/2003
Calvin Smith	8 years experience at Huestis at UO Zebrafish Facility 3 years experience at ZIRC		Zebrafish Facility Staff, ZIRC staff	No	5/2000
Justin Bauer	1.5 years experience at ZIRC		Zebrafish Facility Staff, ZIRC staff	No	12/2007
Evan Williams	1 year experience at ZIRC as a student employee, 2.5 years experience as a ZIRC research assistant		Zebrafish Facility Staff, ZIRC staff	No	4/2005

[1] **NOTE:** Federal regulations require that all personnel involved with animal care and use (including antigen preparation for antibody production) be qualified to perform their duties. Those personnel with any live animal contact must also be a part of the University of Oregon's Occupational Health Program. In order for this animal use application to be approved, the IACUC must have on file training information and qualifications for each individual and documentation of participation in the Occupational Health Program for those individuals with any live animal contact.

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X. **PERSONNEL**[1] Please complete one of these pages for **each** P.I., research assistant, student, postdoc, and **primary** lab employee who will have direct animal and/or tissue contact in this study (including antibody use and antigen preparation for antibody production).

A. PERSONNEL QUALIFICATIONS AND TRAINING

NAME Eri Tallen Campus Phone 346-6028
Position Student Assistant Institute/Department Neuroscience
Work Location ZIRC Emergency Phone 503-537-5085
E-Mail Address etallen@uoregon.edu

In accordance with federal regulations, please provide the following information. **Be advised that the P.I. must assure that all persons participating have demonstrated competence required to perform techniques listed below.**

Indicate Credentials/Background/Experience: None prior to working at ZIRC

Please list those procedures that you will be performing as part of this study: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Please list the procedures and techniques for which you will require additional training: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Training will be provided by: Carrie Barton, Justin Bauer, Calvin Smith

B. OCCUPATIONAL HEALTH PROGRAM (OHP)

___ Not required (No laboratory animal contact.)
X Yes, required - please complete the following information.

Exam Category E: Aquatic Life forms
Physician Review Date: Pending

I have read the protocol and understand my responsibilities outlined therein. I have also read the University of Oregon's Animal Care & Use Training Handbook.

Personnel Signature Eri Tallen Date 7. Apr. 09
~~P.I.~~ Signature Carrie Barton Date 7. April 09
P.I. Signature Zelda Varga 16. April 09

1. NOTE: Federal regulations require that all personnel involved with animal care and use (including antigen preparation for antibody production) be qualified to perform their duties. Those personnel with any live animal contact must also be a part of the University of Oregon's Occupational Health Program. **In order for this animal use application to be approved, the IACUC must have on file training information and qualifications for each individual and documentation of participation in the Occupational Health Program for those individuals with any live animal contact.**

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X. **PERSONNEL**[1] Please complete one of these pages for **each** P.I., research assistant, student, postdoc, and **primary** lab employee who will have direct animal and/or tissue contact in this study (including antibody use and antigen preparation for antibody production).

A. PERSONNEL QUALIFICATIONS AND TRAINING

NAME Tristan Price Campus Phone 346-6028
Position Student Assistant Institute/Department Neuroscience
Work Location ZIRC Emergency Phone 541-912-1878
E-Mail Address tristan@uoregon.edu

In accordance with federal regulations, please provide the following information. **Be advised that the P.I. must assure that all persons participating have demonstrated competence required to perform techniques listed below.**

Indicate Credentials/Background/Experience: None prior to working at ZIRC

Please list those procedures that you will be performing as part of this study: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Please list the procedures and techniques for which you will require additional training: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Training will be provided by: Carrie Barton, Justin Baker, Calvin Smith

B. OCCUPATIONAL HEALTH PROGRAM (OHP)

___ Not required (No laboratory animal contact.)
X Yes, required - please complete the following information.

Exam Category E: Aquatic Life forms
Physician Review Date: Pending

I have read the protocol and understand my responsibilities outlined therein. I have also read the University of Oregon's Animal Care & Use Training Handbook.

Personnel Signature Tristan Price Date 4/7/09
P.I. Signature David Linton Date 4/7/09
Zach Vinyard 10.04.05

1. NOTE: Federal regulations require that all personnel involved with animal care and use (including antigen preparation for antibody production) be qualified to perform their duties. Those personnel with any live animal contact must also be a part of the University of Oregon's Occupational Health Program. **In order for this animal use application to be approved, the IACUC must have on file training information and qualifications for each individual and documentation of participation in the Occupational Health Program for those individuals with any live animal contact.**

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X. **PERSONNEL**[1] Please complete one of these pages for **each** P.I., research assistant, student, postdoc, and **primary** lab employee who will have direct animal and/or tissue contact in this study (including antibody use and antigen preparation for antibody production).

A. PERSONNEL QUALIFICATIONS AND TRAINING

NAME Spencer Noble Campus Phone 415 - 346-6028
Position Student Assistant Institute/Department Neuroscience
Work Location ZIRC Emergency Phone 415-246-9510
E-Mail Address noble@uoregon.edu

In accordance with federal regulations, please provide the following information. **Be advised that the P.I. must assure that all persons participating have demonstrated competence required to perform techniques listed below.**

Indicate Credentials/Background/Experience: None prior to working at ZIRC

Please list those procedures that you will be performing as part of this study: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Please list the procedures and techniques for which you will require additional training: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Training will be provided by: Carrie Barton

B. OCCUPATIONAL HEALTH PROGRAM (OHP)

___ Not required (No laboratory animal contact.)
X Yes, required - please complete the following information.

Exam Category E: Aquatic Life forms
Physician Review Date: Pending

I have read the protocol and understand my responsibilities outlined therein. I have also read the University of Oregon's Animal Care & Use Training Handbook.

Personnel Signature Spencer Noble Date 04/7/09
P.I. Signature Carrie Barton Date 4/7/09
Talia Vega 10/04/05

1. NOTE: Federal regulations require that all personnel involved with animal care and use (including antigen preparation for antibody production) be qualified to perform their duties. Those personnel with any live animal contact must also be a part of the University of Oregon's Occupational Health Program. **In order for this animal use application to be approved, the IACUC must have on file training information and qualifications for each individual and documentation of participation in the Occupational Health Program for those individuals with any live animal contact.**

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X. **PERSONNEL**[1] Please complete one of these pages for **each** P.I., research assistant, student, postdoc, and **primary** lab employee who will have direct animal and/or tissue contact in this study (including antibody use and antigen preparation for antibody production).

A. PERSONNEL QUALIFICATIONS AND TRAINING

NAME Alyssa Jernigan Campus Phone 346-6028
Position Student Assistant Institute/Department Neuroscience
Work Location ZIRC Emergency Phone (719) 210-3219
E-Mail Address jernigan@uoregon.edu

In accordance with federal regulations, please provide the following information. **Be advised that the P.I. must assure that all persons participating have demonstrated competence required to perform techniques listed below.**

Indicate Credentials/Background/Experience: None prior to working at ZIRC

Please list those procedures that you will be performing as part of this study: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Please list the procedures and techniques for which you will require additional training: Maintain and care for zebrafish : feeding fish, cleaning aquariums, breeding/egg production and collection, rearing of embryos, observing fish for illness, removing and recording of dead fish

Training will be provided by: Carrie Barton

B. OCCUPATIONAL HEALTH PROGRAM (OHP)

___ Not required (No laboratory animal contact.)
X Yes, required - please complete the following information.

Exam Category E: Aquatic Life forms
Physician Review Date: Pending

I have read the protocol and understand my responsibilities outlined therein. I have also read the University of Oregon's Animal Care & Use Training Handbook.

Personnel Signature Alyssa Jernigan Date 4/7/08
P.I. Signature Carrie Barton Date 4/7/09
Zoltan Varga 10/04/03

1. NOTE: Federal regulations require that all personnel involved with animal care and use (including antigen preparation for antibody production) be qualified to perform their duties. Those personnel with any live animal contact must also be a part of the University of Oregon's Occupational Health Program. **In order for this animal use application to be approved, the IACUC must have on file training information and qualifications for each individual and documentation of participation in the Occupational Health Program for those individuals with any live animal contact.**

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XI. ASSURANCE STATEMENTS

A. ALTERNATIVES.

The following alternatives must be addressed prior to the use of animals in accordance with Federal policy:

1. Replacement:

I have considered the use of alternatives to the present species, i.e. the use of other species and/or the use of non-animal models and have found them to be unacceptable.

☑ Yes

☐ No

2. Reduction:

I have designed my experimental protocol with careful attention to using the appropriate number of animals and have considered appropriate statistical methods used to reduce the number of animals in this study.

☑ Yes

☐ No

3. Refinement:

I have planned this project to assure that animals are subjected to the minimum amount of pain and distress by the adequate administration of anesthetics, tranquilizers; humane euthanasia; that they receive careful scrutiny of behavioral indices of pain or distress; and that noninvasive imaging technologies are used when appropriate.

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☑ Yes

☐ No

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4. Alternative Methods:

(The following is from USDA Policy #12, June 21, 2000)

Alternatives or alternative methods are generally regarded as those that incorporate some aspect of replacement, reduction, or refinement of animal use in pursuit of the minimization of animal pain and distress consistent with the goals of the research. These include methods that use non-animal systems or less sentient animal species to partially or fully *replace* animals (for example, the use of an in vitro or insect model to replace a mammalian model), methods that *reduce* the number of animals to the minimum required to obtain scientifically valid data, and methods that *refine* animal use by lessening or eliminating pain or distress and, thereby, enhancing animal well-being. Potential alternatives that do not allow the attainment of the goals of the research are not, by definition, alternatives.

The USDA believes that the performance of a database search remains the most effective and efficient method for demonstrating compliance with the requirement to consider alternatives to painful/distressful procedures. However, in some circumstances (as in highly specialized fields of study), conferences, colloquia, subject expert consultants, or other sources may provide relevant and up-to-date information regarding alternatives in lieu of, or in addition to, a database search. When other sources are the primary means of considering alternatives, the Institutional Animal Care and Use Committee (IACUC) and the inspecting Veterinary Medical Officer should closely scrutinize the results. Sufficient documentation, such as the consultant's name and qualifications and the date and content of the consult, should be provided to the IACUC to demonstrate the expert's knowledge of the availability of alternatives in the specific field of study. For example, an immunologist cited as a subject expert may or may not possess expertise concerning alternatives to *in vivo* antibody production.

When a database search is the primary means of meeting this requirement, the narrative must, at a minimum, include:

1. The names of the databases search;
2. The date the search was performed;
3. The period covered by the search; and
4. The key words and/or the search strategy used.

The Animal Welfare Information Center (AWIC) is an information service of the National Agricultural Library specifically established to provide information about alternatives. AWIC offers expertise in formulation of the search strategy and selection of key words and databases, access to unique databases, on- and off-site training of institute personnel in conducting effective alternative searches, and is able to perform no-cost or low-cost electronic database searches. AWIC can be contacted

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at (301) 504-6212, via e-mail at *awic@nal.usda.gov,* or via its web site at *http://www.nal.usda/gov.awic.* Other excellent resources for assistance with alternative searches are available and may be equally acceptable.

Regardless of the alternative source(s) used, the written narrative should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternatives or alternative methods. If a database search or other source identifies a *bona fide* alternative method (one that could be used to accomplish the goals of the animal use proposal), the written narrative should justify why this alternative was not used.

In accordance with the information provided on the preceding page from USDA Policy #12, please provide in the space below a written narrative description of the methods and sources used to determine that alternatives were not available or appropriate for this study.

The Zebrafish International Resource Center seeks to support studies involving the use of zebrafish by providing fish, materials, information, expertise, health services, and disease and husbandry research to the zebrafish research community. Supplying zebrafish cannot be done with out zebrafish. Zebrafish health and husbandry studies require the use of zebrafish to assess the actual effects of proposed experiments. Mutant animals are provided for use in a wide variety of studies (developmental or other) as well as being possibly used in studies of the genetic effects upon disease susceptibility. For experiments requiring experimental tractable genetics, zebrafish is the only vertebrate except mouse where successful genetic analyses are possible and well-developed genetic tools are available.

To some extent, various studies including disease mechanisms can be studied in zebrafish cells growing in cell culture. However, primary cell cultures must be obtained from developing embryos, and ultimately, the predictions of hypotheses about the behavior of embryonic cells in their normal environments must be tested directly in intact systems.

On 04/03/2009, the databases of ASFA (Aquatic Sciences and Fisheries Abstracts), Toxline, Zoological Record Plus, Pubmed, and Google Scholar were searched between the years 1999 and the present for the keywords: zebrafish and disease, (fish) husbandry, microsporidia, mycobacteria, euthanasia, anesthesia, cryopreservation, and toxicity. There were numerous hits on the searches but the articles were not relevant to alternatives to the proposed studies or services.

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B. ASSURANCE FOR THE HUMANE CARE AND USE OF ANIMALS USED FOR TEACHING AND RESEARCH

1. I agree to abide by the University of Oregon policies for the care and use of animals; the provisions of the NIH *Guide to the Care and Use of Laboratory Animals*; and all federal, state, and local laws and regulations governing the use of animals in research. I understand that emergency veterinary care will be administered to animals showing evidence of pain or illness, in addition to routine veterinary care as prescribed for individual species in the Standard Operating Procedures.

2. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified biomedical scientist listed on this protocol.

3. I certify that all personnel having direct animal contact, including myself, have been trained in humane and scientifically acceptable procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project. **I assure that personnel will be allowed adequate time to attend training sessions.**

4. I understand that personnel with live animal contact are required to participate in the Occupational Health and Safety Program.

5. I further declare that the information provided in the accompanying protocol is accurate to the best of my knowledge. Any proposed revisions to the animal care and use data will be promptly forwarded in writing to the IACUC for approval, **including changes in personnel and location.**

6. I am aware that any deviation from an approved protocol or violations of pertinent policies, guidelines or laws could result in immediate suspension of this project.

I have read and understand the assurance statements.

P.I. Signature	MONTE WESTERFIELD, Prof. 4-6-09 Name and Title (typed)
Co-P.I. Signature, if applicable	ZOLTAN VARGA, PH.D Name and Title (typed)

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Department of Veterinary Science
111 Dalrymple Building-LSU
Baton Rouge, Louisiana 70803-6002
(225) 578-4194
Fax: (225) 578-4890
Website: www.lsuagcenter.com

Research and Extension Programs
Agriculture
Economic/Community Development
Environment/Natural Resources
Families/Nutrition/Health
4-H Youth Program

LSU AgCenter Animal Care and Use Committee

April 8, 2009

Dr. Terry Tiersch
Aquaculture

Protocol Number: AE2009-01
Approval Date: 3-03-2009
Expiration Date: 3-03-2012

Protocol **AE2009-01**, entitled "Sperm cryopreservation in small aquarium fishes" lists you as the principal investigator.

The LSU AgCenter Animal Care and Use Committee is happy to inform you that the above protocol was approved. Please contact the committee if any changes in personnel or procedures are made. The IACUC requires that you and any personnel on the project attend the LSU Vet School's Rules and Regulations course within the next six (6) months, if you have not done so in the last three (3) years. The class is held the first Tuesday of every month; please contact Dawn Best-Desjardins (578-9643) to schedule your attendance.

This approval is valid for three (3) years.

Thank you.

Sincerely,

Philip M. Elzer
AgCenter IACUC Chair

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IACUC APPROVED

Hagius, Sue D.

From: Hagius, Sue D.
Sent: Wednesday, March 04, 2009 4:56 PM
To: Tiersch, Terrence R.; Yang, Huiping
Cc: Elzer, Philip H.; Heil, Tammy
Subject: AE2009-01 approval

LSU AgCenter Animal Care and Use Committee

Protocol Number: AE2009-01
Approval Date: 3-03-2009
Expiration Date: 3-03-2012

The protocol, entitled "Sperm cryopreservation in small aquarium fishes," lists you as the principal investigators. The LSU AgCenter Animal Care and Use Committee is happy to inform you that the above protocol has been approved. Please contact the committee if any future changes in personnel or procedures are made. This approval is valid for three (3) years.

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IACUC APPROVED

/12/2009

Revised August 2007

LSU AgCenter PROTOCOL FOR ANIMAL CARE AND USE

PROTOCOL NUMBER: AE 2009 - 01
APPROVAL DATE: 3-03-09

SECTION 1: Principal Investigator

Name: Terrence R. Tiersch	Department: Aquaculture Research Station
Office Phone: (225)765-2848 Home Phone: (225)235-7267	E-mail Address: ttiersch@agcenter.lsu.edu

SECTION 2:

A. Project Title (Enter the name of your project/course number below.)

Sperm cryopreservation in small aquarium fishes

B. Anticipated Project Start Date

03/01/2009. This is a continuing project.

SECTION 3:

A. Animal Species

Species (common name): *Xiphophorus* sp. (swordtails), *Danio rerio* (zebrafish), and *Gambusia* sp. (mosquitofish), *Oryzias latipes* (medaka)	Strain: Assorted strains of each species may be used.	
Total number of animals needed over the 3 year period. (Do NOT count the same animals more than once) TOTAL: 900	Maximum number needed at one time: 200	Number of animals to be placed in each group: 50

No	Are you using wild, invasive, or non-native species for which permits are necessary? (ATTACH COPY) Note: A copy of the permit(s) must be received before animal use begins.

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Revised August 2007

B. Source of Animals

	Order through Agcenter		Other (list source): Texas State Univ. Zebrafish IRC University of Georgia		Transfer from Approved Protocol (list protocol number):

C. Location of Animals

	DLAM Vivarium		Life Sciences Vivarium
	SVM Barns (List Site):		SVM Fish Building
√	LAES (List Site): Aquaculture Research Station		Other (List Site):
	Research Herd		
	Field Study (Do not complete D and E)		

Animal housing and veterinary care have been coordinated with LSU Agricultural Center Unit.

__√__ YES
_____ NO

Name of Animal Housing Representative Contacted (typed): Terrence R. Tiersch

Signature (required): Terrence R. Tiersch

D. Special Husbandry Requirements

Do your animals have special needs to be addressed?

_____ **Animal housing is not required.**
__√__ **NO. Animals will be cared for according to standard operating procedures.**
_____ **YES (complete table below)**

TEMPERATURE RANGE			Humidity: (%)		
LIGHT CYCLE (hours light/hours dark)					
CAGING	Type:	Size:	Filter tops required?		
BEDDING/LITTER	Type:	Autoclaved?	Changes/week:		
WATER	Sterile:	De-ionized:	Acidified:	Tap:	Other:
DIET	Special Feeding Requirements:				
OTHER SPECIAL NEEDS					

Revised August 2007

E. Animal Management

Individual (or groups of) animals are identified by:

___ Tag	___ Tattoo	___ Cage or Stall Card	___ Other... List type of identification:____Tank_____

Check all applicable below:

CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS	PEST CONTROL
___ Call Investigator ___ Clinician to Treat √ Euthanasia	___ Call Investigator ___ Necropsy √ Disposal List any special requirements for disposal:_____ _____	___ Call Investigator ___ Pesticides OK √ No Pesticides

F. Disposition of Animals

What will be done with any animals at the conclusion of the project? Mark all that apply.

√	Animals will be euthanized.
	LAES has permission to REASSIGN animals to another IACUC-approved protocol.
	TRANSFER animals to the following IACUC-approved protocol(s). List Protocol Number(s):_____
	Catch and Release.
	Return to owner/supplier.
	OTHER (please state):_____

Revised August 2007

SECTION 4: Layman's Summary of Research/Teaching

Provide a brief (100 word maximum), **non-scientific explanation of the purpose, materials, and methods** in the block below for the benefit of animal handlers who need to understand the research project.

Xiphophorus, *Gambusia*, *Danio*, and *Oryzias* are small aquarium fishes used in biomedical research. *Xiphophorus* and *Gambusia* are live-bearing fishes. The project will focus on the collection of sperm from males from these species. The sperm will be cryopreserved, thawed and used for artificial insemination of females. The project will examine the effects of cryopreservation on sperm which will be used to fertilize these fishes. This is an important aspect of maintaining these model fishes. The genetic resources of these valuable fishes need to be preserved. Gamete cryopreservation is an effective approach to address these challenges.

SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of The Louisiana State University AgCenter. This project will be in accordance with the USDA "Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching." Agricultural exempt protocols are ones that follow basic animal and veterinary husbandry procedures.

We further assure the Committee that: 1) We will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce significant results are being used in this study.

Principal Investigator Signature — Professor (Title/Rank) — 2/9/09 (Date)

Terrence R. Tiersch
Name of Principal Investigator (typed)

Co-Investigator Signature — Research Assistant Professor (Title/Rank) — 2/9/2009 (Date)

Huiping Yang
Name of Co-Investigator (typed)

Surgeon Signature (if applicable) — Title/Rank — Date

Name of Surgeon (typed)

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Revised August 2007

SECTION 6: Hazardous Materials

If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the IBRDS Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Final approval will not be granted until IBRDSC approval is received by the IACUC. Similarly, if hazardous chemicals are to be used in the animal areas, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL AREA?**

Will Zoonotic Agents be used? ___ YES __√__ NO

List agents:_____

Has request for use of agents been submitted to the Institutional Biological Recombinant DNA Safety (IBRDS) Committee? ___ YES ___ NO

If not, please contact either Dr. Greg Hayes, Biological Safety Manager, at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lsu.edu in the Department of Biological Sciences.

Will Recombinant DNA and/or Virus Vectors be used? ___ YES __√__ NO

List:_____

Has request for use been submitted to the IBRDS Committee? ___ YES ___ NO

If not, please contact either Dr. Greg Hayes, Biological Safety Manager at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lsu.edu in the Department of Biological Sciences.

Will radioisotopes be used? ___ YES __√__ NO

List isotope(s):_____

Are you certified by the Radiation Safety Committee? ___ YES ___ NO

Will hazardous chemicals be used? ___ YES __√__ NO

List compound(s):_____

Please note that approval from Mr. Jerry Steward, Chemical Safety Manager, is required when using hazardous chemicals in the animal facilities. You can contact him at (225) 578-5640 / jsteward@lsu.edu.

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SECTION 7: Animal Treatment Checklist

Check "Yes" or "No" to each of the following questions. Provide an explanation in Section 8 for any "yes" answers. **PLEASE NOTE THAT NOT APPLICABLE (NA) IS AN ACCEPTED RESPONSE.**

YES	NO		Individual(s) Responsible
	√	Will animals be restrained? *(Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)*	Not Applicable
	√	Will animals be fasted?	Not Applicable
√		Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 8. Who will administer? Huiping Yang	Overdose MS-222 will be used for the anesthetizing the fish.
	√	Are neuromuscular blocking agents to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 8. Who will administer agents?	
	√	Will surgical procedures be employed? Are they: Survival_____ Multiple_____ Terminal_____ Who will perform surgery? <u>If survival</u>: **1) Who** will be responsible for recovery of the animals? **2) Who** will maintain post-operative records? **3) Where** will records be maintained?_____ **4) Who** will provide post-op analgesics? **5)** Survival surgeries must be conducted aseptically. Indicate where surgery will be performed.____	
	√	Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)?	Not Applicable

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	√	Is death an endpoint in your experimental procedure? **Note**: *Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.*	Not Applicable
	√	Are there emergency treatments by the DLAM veterinary staff that would not be allowed?	Not Applicable
√		Will animals be euthanized during or at the close of the study? **Who** will perform euthanasia? Huiping Yang and graduate students who will do experiments.	Overdose MS-222 will be used
	√	Will animals be used for antibody production?	Not Applicable
	√	Will Complete Freund's Adjuvant be used? **Must be scientifically justified in Section 8.**	Not Applicable
	√	Will other adjuvants be used? **If yes, please specify.** _____	Not Applicable
√		Will blood be collected? How often? when dissecting the fishes. Volume? 1-2 µL **Who** will collect blood? Huiping Yang and graduate students. **Note**: *Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.*	_____
√		Will live animals be taken from facilities for procedures followed by their return later that day? **If yes, please specify to which areas the animals will be taken:** The laboratory in the next room.	Not Applicable
	√	Will animals be brought onto campus for euthanasia only? **If yes, please specify to which area the animals will be taken:** _____	Not Applicable

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SECTION 8: Summary of Procedures

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a scientific discipline unrelated to yours. Do not use jargon. **Please answer each statement in its own expanding box.**

1. What is the rationale for using animals? Why should this study be done? What hypothesis will be tested?

With the development of genomic research and extensive studies using these fish models, tens of thousands of specific strains and lines have been created and are currently housed worldwide as live animals in resource centers. The genetic resources of these valuable fishes need to be preserved. Gamete cryopreservation is an effective approach to address these challenges. The project will focus on the collection of sperm from males from several species. The sperm will be cryopreserved, thawed and used for artificial insemination of females. The project will examine the effects of cryopreservation on sperm which will be used to fertilize these fishes. This is an important aspect of maintaining these model fishes.

2. Explain how and/or why the particular animal species was selected.

These animals are currently the most widely used fish models for biomedical research.

3. Explain how you arrived at the number of animals to be used (e.g., permitted animal limits in field studies, etc).

Based on the annual research plan. The quantities of fish listed are necessary for experimental replication and also because of the small size of the fish, which results in small volumes of sperm and eggs (10-20 uL) that may need to be combined to provide workable volumes.

4. Provide a complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents (name, dose, volume, route, frequency) that may be administered. If animals are being transported between facilities, describe conditions of transport. Use tables and outlines to indicate group assignments and study progression.

Fish will be held in aquaria with recirculating filtration systems (Aquatic Habitats™, Model ZF0601, Aquatic Ecosystem) according to standard aquacultural practices. Male fish will be killed by overdose of anesthetic (MS-222) and have the testis removed. Female fish will be anesthetized for collection of eggs or for artificial insemination. Females used for these procedures will be returned to the holding systems for recovery. Females may be killed by overdose of anesthetic (MS-222) for collection of embryos. No surgical procedures will be performed on live fish. MS-222 dosage for euthanasia of fish is an immersion in 2 mg/mL (2000 Report of the AVMA Panel on Euthanasia).

5. Describe expected adverse effects. What is the likelihood of these effects?

These animals will be used for sperm collection only, so no adverse effects are expected.

6. Describe procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. For anesthesia and survival surgeries, include a description of post-procedural care and monitoring. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. Include any conditions where veterinary treatment would not be allowed. Specify which treatments would not be allowed, and include a scientific justification. It is advisable that you obtain input from LSU AgCenter's Attending Veterinarian (Dr. Dennis French) or from another veterinarian familiar with the species to be used.

The animals will be cultured in an aquarium system with recirculating filtration systems according to standard aquacultural practices. These fishes will be fed two times each day, and water quality will be monitored once a week. An overdose of MS-222 will be used for anesthetizing the fish before dissection.

7. Describe any euthanasia method to be used. Justify any deviation from AVMA Panel for Euthanasia. The reference is JAVMA 218(5):669-696, 2001, viewable at http://avma.org/resources/euthanasia.pdf.

MS-222 dosage for euthanasia of fish is an immersion in 2 mg/mL (2000 Report of the AVMA Panel on Euthanasia).

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SECTION 9: Type of Project and Narrative Statement

√	**TYPE A** - Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.
	TYPE B - Pain or distress will be relieved by appropriate therapy, e.g. sedatives, analgesics, or anesthetics.
	TYPE C - Drug intervention for pain or distress would interfere with the protocol. **(If this block is checked, specific justification MUST be provided here.)**

Federal regulations mandate that you provide **written, narrative statements** for all projects.

1. **You must state that the proposed activities do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.**

Database used: ISI web of knowledge, and PubMed
Years Searched: Before 2008
Words Searched: Sperm, Cryopreservation, Aquarium fishes, Zebrafish, Medaka, *Xiphophorus*
Date of Search: 1/30/2009
This is a continuing study that does not duplicate previous experiments.
I further assure the Committee that: 1) I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce significant results are being used in this study.

Note: Address the following items (2 and 3) only if you indicated project Type B or C.

2. You must indicate that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Describe any alternatives available and why they are not appropriate.

3. Describe the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

Database used: _____
Years Searched: _____
Words Searched: _____
Date of Search: _____

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SECTION 10: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below.

Name	Rules/Regulations Training Course	Date Attended	Training and Experience**
Huiping Yang	√ Yes ___ No	2/3/2009	√ Yes ___ No
Terrence R. Tiersch	✓ Yes ___ No	3/3/09	✓ Yes ___ No
	___ Yes ___ No		___ Yes ___ No
	___ Yes ___ No		___ Yes ___ No

(p-h) animal [illegible] + class [illegible] DBD

**** The person named has training/experience in assigned procedures for this protocol.**

__Who__ will train individuals for participation in protocol procedures? Answer in block below.

Huiping Yang

Personnel participating in the project must complete the rules and regulations class once every three years. Those who __have not__ attended the Rules and Regulations Course, will have **__six (6) months__** from the approval date of the project to complete them.

Rules and Regulations Courses will be held the __first Tuesday of every month__ from 11:00 a.m. until Noon. Call Ms. Dawn Best-Desjardins at 578-9643 to sign up for these courses.

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SECTION 11: Occupational Health and Safety

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved in the proposed study should participate in the Occupational Health Program administered through the Student Health Center. Currently, there is **no direct cost** for participation in the program. **All persons listed in Section 11 must read the following and indicate level of participation with their signature.**

Occupational Health Program (OHP) - Participation is voluntary, and is open to all personnel with direct or indirect contact with animals used in teaching or research, their bodily products, or materials to which they may be exposed, as described in this protocol. Eligible persons include facility services personnel, animal caretakers, principal investigators, technical staff, graduate and other student workers, and post-doctoral and visiting scientists. All medical information is kept confidential, and is retained by the Student Health Center. You have the right to refuse any and all procedures recommended.

To determine the extent of your participation in the OHP, discuss with the principal investigator named on this protocol, and/or your health professional, any potential physical, chemical, or infectious hazards to which you may be exposed while working on the project. Whether or not you participate, questions related to health risks should be directed to Dr. Tim Honigman, Campus Physician, at the Student Health Center.
If you are at increased risk of illness or injury due to drug-related immune suppression, HIV infection, pregnancy, concurrent illness, musculoskeletal problems, etc., you are advised to discuss your risks with Dr. Honigman, your personal physician, or another health professional.

To participate in the OHP, contact Ms. Dawn Best-Desjardins at 578-9643 for information.

Persons listed in Section 10 above:

Terrence R. Tiersch Printed Name	Signature	___ I choose to participate √ I choose NOT to participate
Huiping Yang Printed Name	Signature	___ I choose to participate √ I choose NOT to participate
Printed Name	Signature	___ I choose to participate ___ I choose NOT to participate
Printed Name	Signature	___ I choose to participate ___ I choose NOT to participate

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Hagius, Sue D.

From: Yang, Huiping
Sent: Wednesday, February 25, 2009 11:41 AM
To: Hagius, Sue D.; Tiersch, Terrence R.
Subject: RE: protocol approval pending minor changes

Sue,

Thank you so much! For the two points you mentioned in the protocol, here are our responses:

1. The use of MS-222 - Is it used to anesthetize the females and kill the males? Do you plan to use it to kill the females too? On page 8, Section 3 "Females may be killed by overdose" – are you saying they might die during the procedure or you will use the MS222 to euthanize them to collect the embryos? Please clarify.
Response: No matter male or females, they will be killed only when it is needed, and only by overdosed MS-222.

2. Page 11, Investigator training – need previous date Dr. Tiersch attended – has 6 months to attend if it has been longer than 3 years…important that this be documented in case of OLAW audit. Please let me know if Dr. Tiersch has attended in the last 3 years (need month and year); if more than 3 years ago, it is very important that he attend and send me the date when he does so. For the approval, I need the previous date.
Dr. Tiersch will attend the training next week (March 3, 2009).

Thanks, and any other question, please let us know.

Huiping

From: Hagius, Sue D.
Sent: Wednesday, February 18, 2009 3:36 PM
To: Tiersch, Terrence R.; Yang, Huiping
Cc: Elzer, Philip H.; Hagius, Sue D.
Subject: protocol approval pending minor changes

Tiersch/Yang

LSU AgCenter Animal Care and Use Committee

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Protocol Number: AE2009-01
Approval Date: 2-13-2009, pending
Expiration Date: 2-13-2012

The protocol, entitled ""Sperm cryopreservation in small aquarium fishes," lists you as the principal
ıvestigators. The LSU AgCenter Animal Care and Use Committee is happy to inform you that the above protocol was
ıpproved at the last meeting, pending some minor changes (see below). Final approval will be issued once these
ıodifications are received. Please contact the committee if any future changes in personnel or procedures are made.
he IACUC requires that you and any personnel on the project attend the LSU Vet School's Rules and Regulations
ɔurse within the next six (6) months, if you have not done so in the last three (3) years. The class is held the first
uesday of every month; please contact Dawn Best-Desjardins (578-9643) to schedule your attendance.
This approval is valid for three (3) years.

/2/2009