

**REVIEW PROTOCOL****INDIANA UNIVERSITY OF PENNSYLVANIA  
Institutional Animal Care and Use Committee (IACUC)**

- All information must be typed. Handwritten proposals are not accepted.
- Submit one hard copy with original signatures to: Institutional Animal Care and Use Committee, Office of the Dean, College of Natural Sciences and Mathematics, 305 Weyandt Hall

*Please click in the shaded fields to complete form*

**1. Principal Investigator:**

Name **John Green**  
Email address **jgreen@iup.edu**  
Campus address **326 Weyandt Hall**  
Department/Division **Biology**  
Status:  Faculty  
 Staff  
 Doctoral candidate  
 Grad student  
 Undergrad  
 Other

**2. Students please provide the name of faculty supervising your project:**

Faculty Advisor \_\_\_\_\_  
Department \_\_\_\_\_  
Position/Rank \_\_\_\_\_  
Email address \_\_\_\_\_  
Daytime Phone \_\_\_\_\_  
Campus address \_\_\_\_\_

**3. Project Title** **The effect of various stressors on the metabolism of fish**Date of submission **2-28-11**Dates during which project will be conducted – From **2-1-11** To **1-31-12****4. Project Funding Source** (check as many as apply)

Extramural Grant – Agency name \_\_\_\_\_  
 IUP Grant  
 Non-funded faculty or student research  
 Other

**5. Purpose** (check one)  Teaching (Course \_\_\_\_\_)  
 Research**6. Species** **Pimephales promelas and Gambusia halbrookii**Number of subjects **2200 for each species** Source of Animals **Hatcheries (Jones and Zetts)**

7. Please discuss the following information as it relates to your use of animals for research or teaching. Information should be discussed at a level suitable for a layperson but without omission of relevant information. Responses to the following questions should be typed and submitted with the previous cover page.

- A. Purpose of the Study – State concisely what the study is intended to accomplish.

The influence concentration of heavy metals, specifically copper, and time of exposure have on metabolism in aquatic organisms is still poorly understood. The purpose of this study is to determine how fish are affected by acute and chronic exposure to a stressor such as copper.

- B. Background – Briefly state the background and rationale for your study, including some relevant references. Identify the main questions the proposed research is designed to address.

Aquatic ecosystems, especially their biological assemblages, continue to be degraded globally. The two most important problems affecting water in Western Pennsylvania are acid mine drainage (AMD) and heavy metal pollution (Han and Kim, 2009). Streams contaminated by AMD often have a decreased abundance and diversity of fishes and are characterized chemically by low pH, high sulfate and metals concentrations, and high conductivity (Raymond and Oh, 2009). Copper from mines and cadmium from various types of modern industrialization are especially toxic to fish species. Effects on fish include disturbances in their metabolic processes, osmoregulation, respiration and tissue damage (Austin, 1999). One of the biggest problems in trying to determine how various stressors such as pollution affect fish is the lack of consistency in the methods used to determine acute and chronic toxicity. One author may study copper and use four days for a chronic exposure while another may study cadmium and use three weeks for a chronic exposure. Three years ago, I began collaborating with Dr. Ed Farley from Penn State University to address this issue. We published a paper that was the first to look at multiple stressors in a species with similar acute and chronic exposures. We are expanding this original study into different populations of fatheads.

- C. Characteristics of the Subject Population – Provide the following information regarding your subject population.

- a. Species Pimephales promelas (fathead minnow) and Gambusia holbrooki (eastern mosquitofish)
- b. Age Range and/or weight range Adults between 1 and 2 grams for both species
- c. Sex Both male and females will be used
- d. Why does this project require the use of animals (i.e., could results be obtained in another manner such as computer simulation)? We require animals for this experiment because we are testing the effects of stressors such as copper on metabolic rates of fathead minnows and mosquitofish. The EPA has designated fatheads as a model organism in aquatic toxicology studies (EPA Fathead Minnow acute toxicity database).
- e. Why does the project require the use of this particular species? Since initial studies with the species in the 1950s, the fathead minnow has become the most widely used small fish model for regulatory ecotoxicology in North America (Ankley and Villeneuve, 2006). Mosquitofish have been used as a bioindicators species for pollution since the 1970s (Pyke, 2005).
- f. Why does the project require this number of animals? Justify the number of animals needed for your experiments. Please provide this information in tabular form (e.g., numbers of experimental groups x sample size of each group = total number of animals requested). There are three parts to this study: (1) range finding (upper non-lethal level of Cu); (2) acute and chronic exposure; and (3) determination of heterozygosity . (1) 24 experimental regimes x 5 sample size = 120; (2) 4 populations x 2 = 8 experimental regimes x 60 sample size = 480; (3) 4 experimental regimes x 400 sample size = 1600. Total of 2200.

- g. Does the Biology Department/Field Station have the proper facilities to maintain the animals, meeting USDA Animal Welfare regulations as to housing, food and water? **Yes – the Biology Department through an NSF grant created a wet lab in room 4 of Weyandt. It contains a walk-in environmental chamber with temperature and photoperiod control and three frigid unit temperature controlled 500 gallon tanks.**
- h. At the conclusion of this project what will be done with the animals? If they will be euthanized complete section 12. **At the end of experiments 1 and 2, fish are released in local streams (they are native to this area). We work with Yellow Creek Park Manager to release them in streams of his choice. In the third experiment fish will be euthanized by immersion in > 250 mg/L concentration of MS222. Fish will be left in this solution for at least 10 minutes following cessation of opercular movements before being frozen. These are the recommended guidelines in two major publications: (1) Guidelines for the Use of Fishes in Research (2004), American Fisheries Society; and (2) American Veterinary Medical Association Guidelines on Euthanasia (2007).**

**8. Methods and Procedures – Provide the following information regarding your methods and procedures.**

- A. Describe your experimental design. (Do not address surgical procedures here. Surgical procedures are to be described in item 9). Include in your description everything that is done to the animal. **The methods for doing the three experiments are given in various publications of the EPA such as Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition, Short-term Methods for Estimating the Chronic Toxicity of Effluents, Receiving Water to Freshwater Organisms and Fish acute toxicity test, Guideline 203, OECD Guidelines for Testing of Chemicals, Section 2 (1992). There is also a Fathead Minnow Acute Toxicity Database for further reference. Finally, these protocols were summarized in the book Fundaments of Aquatic Toxicology: Effects, Environmental fate and Risk Assessment (Editor: Gary Rand, 2003). A brief description for each follows.**

Adult fathead minnows (*P. promelas*, Cyprinidae) will be obtained from four different hatcheries (Zett's, Jones, Hilltop, and Schultz's). Adult mosquitofish will be obtained from two different hatcheries (Jones and Hilltop). Both species will be acclimated for two weeks in a 1500-L holding tank containing dechlorinated tap water at 18 °C with constant aeration. Fish will be maintained on a 12L:12D photoperiod and fed to satiation every other day using commercial flake fish food.

Experiment 1 – Range finding tests. These tests are used to determine the upper non-lethal levels of Cu. These levels are based on the highest concentration of copper at which no mortality is observed over a 96-h period. Nominal concentrations of Cu are obtained from a stock solution (100,000 µg·L<sup>-1</sup> CuSO<sub>4</sub> pentahydrate). Thirty total fish of each species are used with five each placed into six 57-L aquaria at 18 °C containing dechlorinated tap water and a specific concentration of copper (25, 50, 100, 150, 200, and 500 µg·L<sup>-1</sup> Cu). This will be repeated for each of the four populations of fatheads and two populations of mosquitofish we will study (30 X 4 = 120 fish).

Experiment 2 – Fish (n=15 for each exposure regime) of each species are exposed for either 24h or 96h to one of three nominal concentrations of Cu in a 56.7L tank. Nominal concentrations in previous studies were 0 (control), 60, 90, and 120,µg·L<sup>-1</sup> Cu. Thus, four populations, each with an acute and chronic exposure, use 45 experimental and 15 control fish for a total of 480 fish of each species. Aquaria are provided with aeration throughout the exposure period and, in the case of 96-h exposures, one-half of the volume of water with a similar level of copper will be replaced at 48 h. Fish are fasted for 24h before exposure and are not fed throughout the experiment (24h) or after 48h

(96h). Body mass and metabolic rate will be determined on each individual at the beginning and end of each exposure. Metabolic rate is determined by placing the individuals in 150-mL polypropylene bottles with a magnetic stirrer. A Strathkelvin 782 oxygen meter and 1302 dissolved oxygen electrode are used to measure oxygen consumption. Oxygen consumption values are used to calculate metabolic rate as ml O<sub>2</sub>·g<sup>-1</sup>·hr<sup>-1</sup>.

Experiment 3 – In order to determine the heterozygosity in each population, a total of 400 fish from each hatchery will be used (1600 total fatheads and 800 total mosquitofish). Twenty tanks (56.7L) with 20 individuals per tank (20 tanks) will be exposed to 50% of the upper non-lethal limit of Cu for 96h for each species. Similar procedures as outlined in experiment 2 will be used for feeding and water changes. Metabolic rates and body mass will be taken at the beginning and end of each exposure using the same procedures outlined in experiment 2. After the final exposure fish will be euthanized by freezing and transferred in liquid nitrogen to Dr. John Peles laboratory at Penn State for the genetic studies. There are no controls necessary for this experiment as it is only a correlation between the metabolic rate and the amount of heterozygosity in the organism.

- B. Does this project duplicate previous work? If so, what is the justification for duplication (e.g., teaching, verification of results, etc.)? If a literature search was performed to rule out duplication of previous work, please give the date of the search, keywords that were used to perform the search, period covered by the search, databases searched, and results of the search. Elevent peer-reviewed papers have been published regarding the effects of copper on the metabolism of fat-head minnows. Another 76 peer-reviewed papers have been published on some aspect of copper exposure in fathead minnows. I used the Ebsco host search with the key words fat-head minnow and copper and metabolism as well as fat-head minnow and copper. Using the same database we found that there were four peer-reviewed papers using the search words mosquitofish and copper with one of those papers dealing with metabolism. Another 61 peer reviewed papers were found when using the key words mosquitofish and metabolism. This project does not duplicate previous work but rather expands on an earlier project by myself and Rick Gray. The main issue that separates our work from the other publication sis that there has not been a clearly defined time period for short and long term exposure until our work.
- C. State why the animal model that you selected is appropriate for your experiments. Fathead minnows are the fish species chosen by the EPA for use in toxicology studies. Mosquitofish have been used as biological indicators for pollution for over 30 years. Both species are also easily obtained from hatcheries.

## 9. Surgical Procedures

- A. Is surgery to be performed on the animal(s)? If “No” proceed to next item. If “Yes” please complete the rest of this section. No surgeries will be performed on the fish.
- B. Please indicate whether this is a survival surgery or a non-survival surgery. \_\_\_\_\_
- C. Please state where the surgical activity will take place (e.g., lab, room number, etc.). \_\_\_\_\_
- D. Describe the surgical procedure. \_\_\_\_\_

- E. Will anesthetic, analgesic, or tranquilizer drugs be administered? If so, by whom, when, and in what dosages? \_\_\_\_\_
- F. Describe the post-surgical or post-treatment monitoring and care procedures, including all drugs to be used, their dosages, and routes of administration. \_\_\_\_\_
- G. Will more than one survival surgery be performed on the same animal? If "Yes" please justify. \_\_\_\_\_

## 10. Pain

- A. Choose the description listed below which most closely describes the potential for discomfort or pain as a result of the experiment.
  - a. This procedure should cause only minor or no pain or distress. **X**
  - b. This procedure can cause moderate pain and/or distress. However, appropriate anesthetic, analgesic, or tranquilizer drugs will be administered to eliminate or minimize pain and/or distress. Please provide a brief summary of drugs, doses, routes of administration and frequency of administration.  
The procedures we will be using are standardized by the EPA so no alternatives needed to be considered. The methods for doing the three experiments are given in various publications of the EPA such as Methods for Measuring the Acute Toxicity of effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition, Short-term Methods for Estimating the Chronic Toxicity of Effluents, Receiving Water to Freshwater Organisms and Fish acute toxicity test, Guideline 203, OCED Guidelines for Testing of Chemicals, Section 2 (1992). There is also a Fathead Minnow Acute Toxicity Database for further reference. Finally, these protocols were summarized in the book Fundamentals of Aquatic Toxicology: Effects, Environmental fate and Risk Assessment (Editor: Gary Rand, 2003).
  - c. This procedure can cause moderate pain and/or distress. However, no anesthetic, analgesic, or tranquilizer drugs will be administered to minimize pain and/or discomfort. Please justify. \_\_\_\_\_
- B. Federal guidelines require documentation that you have considered alternatives to procedures that cause more than momentary or slight pain (e.g., tissue culture, computer simulation, etc.). If a literature search was performed, please give the date of the search, keywords that were used to perform the search, period covered by search, databases searched, and results of the search. \_\_\_\_\_

## 11. Potential Hazards

- A. Are any of the agents used in this project hazardous (Yes, No, Unknown Hazard)? If you plan to use a hazardous agent(s), list the agent(s) below in the appropriate category.
  - a. Carcinogens \_\_\_\_\_
  - b. Highly toxic compounds \_\_\_\_\_
  - c. Infectious agents \_\_\_\_\_
  - d. Radiation, radioisotopes \_\_\_\_\_

If you plan to use a substance(s) that constitutes one of the above hazards or an unknown hazard, please describe procedures to minimize risks to the animals, yourself, and others. Copper – Federal regulatory Limit: action level for copper is 1.3 mg/L (PA has same limits). Since we are well below the action level (micrograms) no specific action needs to be taken in our disposal of the water containing copper.

## 12. Euthanasia

- A. Describe the method of euthanasia at the conclusion of the experiment. Please include drugs, dosages, and routes of administration, if appropriate. In the third experiment fish will be euthanized by immersion in > 250 mg/L concentration of MS222. Fish will be left in this solution for at least 10 minutes following cessation of opercular movements before being frozen. These are the recommended guidelines in two major publications: (1) Guidelines for the Use of Fishes in Research (2004), American Fisheries Society; and (2) American Veterinary Medical Association Guidelines on Euthanasia (2007).
- B. After euthanasia, please state what method will be used for disposal of the carcass. \_\_\_\_\_
- C. What is the end point at which you have determined that an animal will be euthanized for human reasons (i.e., percentage of weight loss, general appearance, etc.)? If death is chosen as an endpoint, please justify. \_\_\_\_\_

### 13. Experience of Investigators

- A. Describe the Principal Investigator's training and experience in relation to this project. If this project has co-investigators, list them and describe their training and experience. I have been working with this system extensively over the past three years. There has been one publication and three posters at a North American meeting on our results. The latest poster was given at the 31<sup>st</sup> Annual Meeting of SETAC (Society of Environmental Toxicology and Chemistry), November 8, 2010, in Portland, OR.

### 14. Permits

- A. Are any state, federal, or other permits required for the proposed research?

\_\_\_\_\_ Yes       No

- a. Describe the permits that are required and identify the agencies with authority to provide such permits.  
\_\_\_\_\_
- b. Attach copies of approved permits.

### 15. Sponsor Statement for Student Sponsored Research – For experiments being conducted by an undergraduate or graduate student, please have your advisor for this project address the following issue:

- A. Please describe the degree of supervision and training that you will provide for students working on this experiment. \_\_\_\_\_

### 16. Signature of Principal Investigator

By signing below you are agreeing to the following statements. I agree to provide whatever supervision is necessary to ensure the welfare of the animal subjects being used. I understand that I cannot use animal subjects until I have received approval from the Institutional Animal Care and Use Committee (IACUC). I understand that as the principal investigator I am ultimately responsible for the welfare and protection of animal subjects and will conduct this project as approved. Any additions to or changes in procedures as well as any problems associated with the use of animal subjects must be reported to the IACUC.

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Signature of Principal Investigator

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Date

**17. Signature of Faculty Sponsor (if applicable)**

I affirm the accuracy of this application and accept responsibility for the conduct of this research and supervision of animal use.

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**Signature of Faculty Sponsor**

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**Date**