Teaching Stress Physiology Using Zebrafish (*Danio rerio*)

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Abstract: A straightforward and inexpensive laboratory experiment is presented that investigates the physiological stress response of zebrafish after a 5 °C increase in water temperature. This experiment is designed for an undergraduate physiology lab and allows students to learn the scientific method and relevant laboratory techniques without causing significant stress to animals. An additional experimental design and a set of additional questions for lab report are also included.

***Keywords*:** zebrafish, temperature, stress, physiology lab

Introduction

The use of hands-on experiments with animals is an important method of teaching undergraduate biology students about physiology. However, there has been a recent trend of using computer-simulated experiments to teach physiology to students. Although there are numerous benefits to using computer technology, simulated experiments cannot replace the experience of working with animals such as mice, frogs, and zebrafish. Zebrafish have been used to study vertebrate development and genetics, and as a model for studying human disease, aging, oxidative stress etc. (see review by Gerhard and Chang, 2002). Zebrafish have also been used in studying developmental physiology (Barrionuevo and Bufggren, 1999) and there are studies on the effects of heat shock at the molecular level (Krone *et al.*, 1997; Yabu *et al*., 2001). However, the studies related to normal basic physiology of zebrafish are limited. Given that zebrafish are inexpensive and easy to maintain we wanted to explore the possibility of using them to teach physiology in an undergraduate lab. This paper describes an experiment using zebrafish as a model to teach students about the stress response in vertebrates.

Background

The stress response in fish has many similarities to that of terrestrial vertebrates (Wendelaar Bonga, 1997). By studying the stress response in zebrafish, students can apply what they learn to other vertebrates. According to Hans Selye, a renowned physician, a stressed organism passes through three stages that make up the General Adaptation Syndrome (GAS). The first stage is an alarm reaction in which the neuroendocrine system is activated and stress hormones are produced. The second stage, adaptation, which generally occurs within 24-48 hours, involves the organism's return to the pre-stress state or an altered resting state through physiological changes (i.e., increased respiration, decreased metabolism, and decreased immune response). If the stress is too severe or chronic, adaptation may not be possible and the organism enters the third stage, exhaustion, which affects the entire organism. During the third stage, consequences such as poor growth, poor reproduction, and increased susceptibility to diseases may be observed (Barton and Iwama, 1991).

There are many parameters that can be used as indicators of stress in fish (Barton and Iwama, 1991; Wedemeyer et. al. 1990). In this experiment, five parameters including opercular beats (ventilation frequency), oxygen consumption, blood glucose, spleen somatic index (SSI), and condition factor (K) were used to study the effects of temperature stress. Ventilation frequency is an indirect measure of the metabolic rate of fish. Fish initially adapt to stress by increasing their ventilation frequency. If this adaptation is successful, other physiological changes may not occur. Stressed organisms usually require more oxygen to carry out their metabolic processes. In order to compensate for the increased oxygen requirement during stress, hyperventilation may occur. Blood glucose is another indicator of metabolic stress, and functions to provide the caloric energy needed for a fight-or-flight response. Blood glucose levels become elevated due to the action of the stress hormone, cortisol. The spleen stores red blood cells and releases them for circulation, and can decrease in size during stress. The condition factor indicates the overall health of fish. Stress can affect the condition factor of fish by causing them to have a smaller than usual weight at a particular length. As such, the condition factor reflects the growth or wellbeing of an individual fish. Typically, the condition factor is one of the last parameters to change when a fish is under stress, and the change usually occurs in the exhaustion stage.

It is well known that a significant increase or decrease in water temperature can cause physiological changes in vertebrate animals that may lead to the exhaustion stage of the stress response. However, various experiments have shown that a 2-5 °C increase in water temperature does not result in any significant physiological changes (Burka et al., 1998). Hence, we wanted to use a fish model system and temperature stress to teach physiology.

Hypothesis

The present study was used to investigate the effects of a 5 °C temperature increase on zebrafish with the hypothesis that no significant changes would occur in the following physiological parameters: opercular beats (ventilation rate), oxygen consumption, blood glucose, spleen somatic index, and condition factor.

Goals

Our goals for conducting the experiment described in this paper were to make sure that zebrafish are not stressed to exhaustion under the experimental conditions and to ascertain the feasibility of using such an experiment in an undergraduate physiology lab by allowing undergraduate students to conduct the experiment, collect and analyze data and present their finding in a professional setting.

Learning objectives/ outcomes

After completing the exercises presented and suggested in this paper the students should: (1) understand basic concepts and principles related to stress physiology, (2) be able to define common physiological terms and describe the parameters used to evaluate stress, (3) formulate and test the hypothesis, (4) gain experience in the laboratory techniques used during the experiment, (5) demonstrate an ability to collect, analyze and interpret data, and (6) be able to describe experimental design and present their results and analyses in a written or an oral form. Instructors might want to use Bloom's taxonomy or Revised Bloom's Taxonomy to assess student learning as described by Forehand (2001).

Materials and Methods

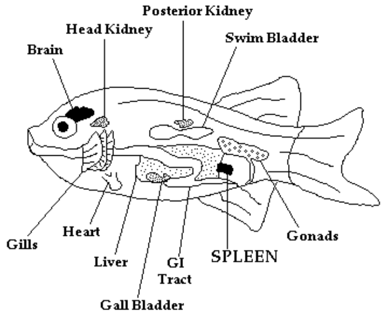
Sixty zebrafish were purchased from Aquarium 33 in Fort Wayne, IN, divided into two groups (control and experimental, each with 2 replicates), and placed randomly into four 20 gallon glass aquaria which contained dechlorinated water. The dechlorination process involved filling large bins with city water, which remained uncovered and aerated for 2-3 days. This process enabled the slow evaporation of chlorine from the water sources. The aquaria water was filtered and oxygenated by the use of Millennium 2000 filters (Aquarium Systems, Mentor, OH). Water within the aquaria was heated to an optimum temperature of 28 °C by the use of Visi-Therm Deluxe 100W heaters (Aquarium Systems, Mentor, OH) and fish were acclimated for 2 weeks. After the acclimation, water temperature in the experimental aquaria was raised to 33 °C. The fish were fed TetraMin: Large Tropical Flakes (The Rich Mix) (Tetra, Melle, Germany) twice daily to satiation and cared for according to the guidelines of the appropriate animal care committee.

Stress parameters were measured from randomly sampled fish (6 fish per group: 3 from each replicate) on days 0 (before the water in the experimental aquaria was heated), and on days 2, 4, 6, and 8. For measuring the rates of opercular beats and oxygen consumption, each sample fish was placed in a glass beaker with pre-measured water, each beaker was equipped with an YSI Oxygen Probe (YSI Incorporated, Yellow Springs, OH) to measure the oxygen levels, and sealed with Parafilm to minimize any addition or loss of oxygen. Because parafilm does not totally prevent exchange of gases, the measurements were taken for a very short time (3 minutes) and averaged. Also, the conditions used for oxygen measurement were identical for both control and experimental groups hence the relative values are still valid. The reason for the simple set up is to make it possible to perform it in an average undergraduate laboratory where sophisticated apparatus may not be available. The opercular beats were then recorded using a digital video camera, and the levels of oxygen were measured using the oxygen probe over 1 minute. The video was later used to count the number of times the operculum opened and closed per minute. The oxygen consumption rate was calculated by subtracting the final O_2 concentration from the initial O_2 concentration per minute. After these 2 parameters were measured, the zebrafish were euthanized by qualified personnel using a lethal dose of MS-222 (greater than 200 mg/L) (Wedemyar et al., 1990).

Condition factor (K), was measured by taking the length (L) and weight (W) of each zebrafish and using the following formula: $K = (W \times 100) / L^3$ (Ibrahim et al., 2000). A drop of blood from each fish, from the severed caudal peduncle, was then obtained and placed on a glucose strip in a standard glucometer (Prestige Smart System, Home Diagnostics, Deer Field, IL) to determine the levels of blood glucose. Directions from the manufacturer were followed when operating the glucometer. This method has been validated for use in glucose analysis for fish (Wedemyar et al., 1990). After blood determinations were concluded, each fish was carefully dissected to obtain the spleen (Figure 1). The spleen somatic index (SSI) was calculated using the formula: ((spleen weight

(g) / body weight (g)) x 100 (Garcia-Abiado et al., 2004).

Figure 1. Zebrafish anatomy



After the sampling period ended, statistical analysis of the data was performed. The means and their standard deviations were calculated for each parameter. Analyses were carried out using Student's t-test with Minitab (Minitab ® Release 14 2006). Differences were considered significant when $p < 0.05$.

Results and Discussion

Data for opercular beats is presented in Figure 2 which shows that at days 2, 4, 6, and 8 there was a significant increase in the number of opercular beats. The results for oxygen consumption, blood glucose levels, spleen somatic index, and condition factor are presented in Table 1. There were no significant differences between the control and experimental groups of fish except for glucose at day 4. The difference in blood glucose at day 4 was due to an outlier or anomaly in the data and hence we conclude that the difference was not related to the stress response.

Figure 2. Number of opercular beats for control and experimental zebrafish. * Significantly different from control (p <0.05).

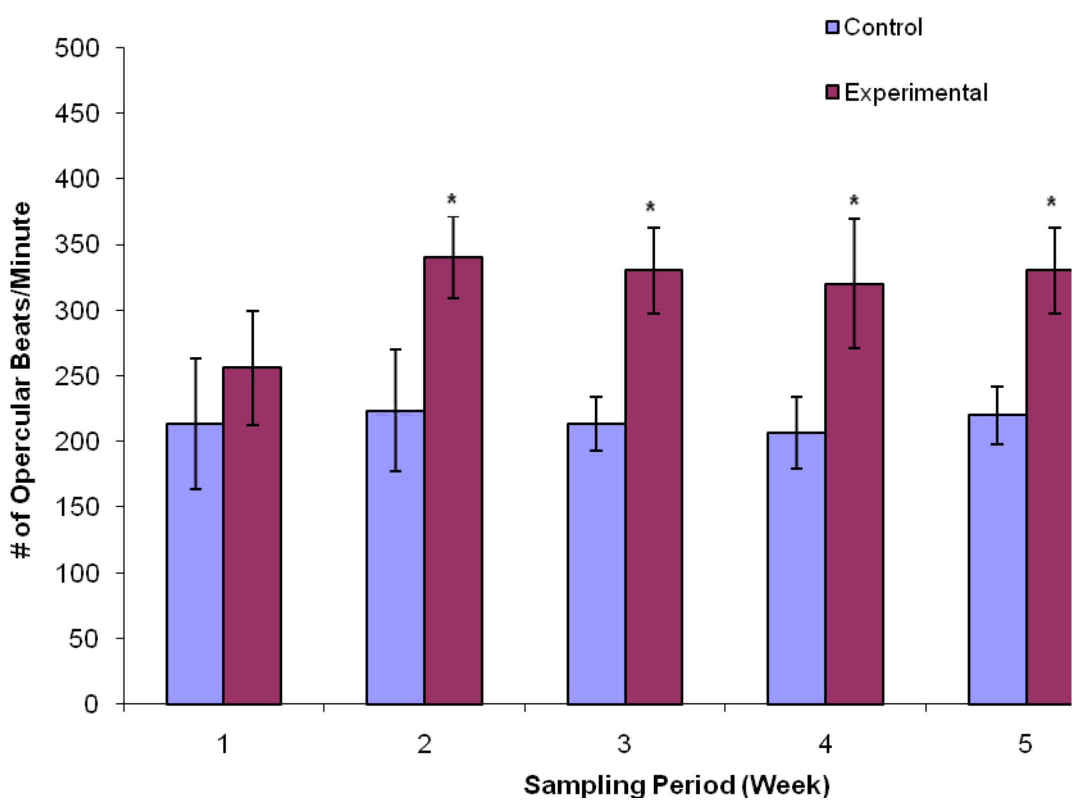


Table 1. Comparison of stress parameters between two groups (control and experimental)

Fish Group (n = 6)	Sampling Period (Days)	Oxygen Consumption (mg/L /min)	Blood Glucose (mmol/dL)	Spleen Somatic Index (SSI)	Condition Factor (K)
Control (28 °C)	0	0.86±0.27	28.00±9.66	0.07±0.03	0.90±0.25
Experimental (33 °C)	0	0.71±0.16	44.00±18.01	0.10±0.06	0.82±0.19
Control (28 °C)	2	1.30±0.74	58.75±31.68	0.11±0.07	0.86±0.11
Experimental (33 °C)	2	1.61±0.33	59.66±28.51	0.10±0.05	0.89±0.10
Control (28 °C)	4	0.89±0.16	25.25±9.91	0.15±0.10	0.99±0.10
Experimental (33 °C)	4	0.94±0.16	61.60±22.91*	0.10±0.04	0.94±0.12
Control (28 °C)	6	1.34±0.17	15.75±5.73	0.14±0.03	0.96±0.18
Experimental (33 °C)	6	1.24±0.30	25.66±11.02	0.14±0.04	0.93±0.06
Control (28 °C)	8	1.00±0.19	34.50±25.09	0.15±0.05	0.90±0.10
Experimental (33 °C)	8	1.03±0.19	42.66±24.68	0.16±0.10	0.90±0.22

* Significantly different ($p < 0.05$); means ± standard deviation

A significant part of stress adaptation involves the redistribution of metabolic energy away from activities such as growth and reproduction and towards activities such as respiration that will help to restore homeostasis. The data presented in Table 1 and Figure 2suggest that in order for the zebrafish to successfully adapt to the 5^0C temperature increase from the optimal temperature (28^0C), they reallocated some metabolic energy to increase their ventilation frequency. Some increase in oxygen consumption was also observed but it was not statistically significant. Hence, we suggest that because the zebrafish were able to adapt by increasing their opercular beats (Figure 2), more significant physiological changes such as an increase in blood glucose or reduction in spleen size did not occur and movement into the exhaustion stage was prevented. Thus, there was no change in the condition factor which is affected only in the third stage of the stress response. Since no significant physiological stress occurred when the temperature was increased by 5^0C (above optimal temperature 28^0C), concerns about exposing animals to stress were eliminated.

Given that this experiment was performed by three undergraduate students, we feel that it is well-suited for undergraduate physiology labs and will enable students to develop and test hypotheses, describe an experimental design, learn lab techniques, collect and analyze data, draw conclusions, and present results as they relate to the stress response. Before students perform the experiment the instructor may divide students into five groups and provide them with the references for five parameters to be tested (the references are given in this manuscript) and instruct students to develop a hypothesis and an experimental design to test the hypothesis. It is likely that their design will be different from what we have proposed below. This will provide an opportunity for the instructor to discuss pros and cons of their design. The instructor may use their design (if practical and appropriate). After the experiment is completed the students can be asked to give an oral presentation or write a report in a scientific format. In either case, assessment can be based on content, synthesis, data interpretation, sequence and organization of material, clarity. For oral presentation additional criteria such as addressing audience well and handling questions can be added. The instructor might want to use the Likert scale of 1-5 with 5 being excellent.

Although this experiment was conducted over an eight day period in the classroom setting, the experiment may be performed over a different period of time (i.e., five weeks) at the instructor's discretion. Also, if an instructor wishes to devote only one laboratory session to this experiment, we suggest the following alternative experimental setup:

1) The instructor or lab personnel will have to set up 10 tanks 10 days prior to the day of actual experiment to be performed by the students. Out of these 10 tanks, use 5 tanks for the control group and 5 tanks for the experimental group. Raise the temperature in 1 experimental tank to 33 °C every 2 days. Therefore, at the end of 10 days, there will be 5 tanks each representing a time point (2, 4, 6, 8, and 10 days of exposure to increased temperature).

2) On the tenth day divide student into 5 groups and give each group one control and one experimental tank. Have each group collect data for all 5 parameters from their control and experimental tanks during the same lab period.

3) Pool the data from each group and distribute it to the class – thus each student will have data for all five time points.

4) Have each group perform data analysis using statistical software.

5) Require students to write a lab report and present results, interpretations, and conclusions in the desired format.

It is also important to keep in mind that the animal care protocol chosen should be approved by an animal care usage committee prior to beginning the experiment. If desired and time permits, students can be made aware of the regulations related to the use of animals in the laboratory. This is particularly relevant to the experiments that might be perceived as cruelty to animals by subjecting them to stress. It may also be used as an issue worth discussing in class.

Furthermore, students can be assigned to answer additional questions in their lab report such as:

1) Is it possible that genetics plays a role in the stress response of zebrafish? Would it be better to conduct this experiment with an isogenic pool of zebrafish or a wild population?

2) Would an instantaneous increase in water temperature yield different results?

3) Ventilation frequency (opercular beating) consumes energy. If stress increases the rate of ventilation, would this loss of energy begin affecting other parameters over time?

4) Does water temperature affect the amount of dissolved oxygen? If so, would an increase of 5 °C significantly impact the oxygen consumption results?

5) Can the same question be answered using an alternative experimental design?

6) What other hypotheses involving zebrafish and stress physiology could be investigated?

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