The Journal of Toxicological Education **1:** 10-20 (2013)
http://www.jtoxed.org

# A Toxicological Study using Zebrafish (*Danio rerio*) as a Model

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**Abstract**

Only recently has it been adequately recognized that substances present in the environment can have adverse effects on developing organisms. Now, with environmental pollutants accumulating at an unprecedented rate, and with pharmaceuticals dominating western medicine, it is particularly important that we understand the effects of the substances to which we are exposed. Zebrafish (*Danio rerio*) has become a widely used model system for the study of vertebrate development. This system is particularly amenable for use in the undergraduate laboratories because of the ease of collection and manipulation and the rapid rate of development. In this lab, students use zebrafish to examine the effects of nicotine, ethanol, and retinoic acid on normal development. Students first examine normal development and compare it to overall growth, dry weight, and behavior of zebrafish exposed to these chemicals. The students may also collect data on $LC_{50}$ and notochord length. The quantitative data is evaluated for statistically significant differences between treatments. Finally, students write a research proposal for an independent experiment in which they expose embryos to a toxicant of their choice, carry out the experiment, and present their findings. This lab introduces students to the use of animal models and incorporates experimental design and data analysis. More importantly, it introduces students to concepts and models in toxicology to increase their awareness and interest.

**Keywords**

Zebrafish; toxicology, education; laboratory study

## Introduction

There has been an increase in the demand for new, robust, and cost-effective ways to assess chemicals for their effect on human health, particularly early development. This is important because of the increase in the amount of chemicals produced and released into the environment and the threat for potential exposure. Many mammalian models for toxicology are expensive and difficult to manipulate and assess during embryonic stage. Within the past decade, the zebrafish has become an important model organism for toxicological research[1].

Zebrafish, *Danio rerio,* are vertebrate organisms that are an excellent alternative model due to their small size, rapid external development, optical transparency during early development, permeability to small molecules, genetic similarity to humans, and great fecundity. Zebrafish embryos develop most of the major organ systems present in mammals, including the cardiovascular, nervous and digestive systems in less than a week [1-3]. Zebrafish are also an excellent biomedical model because 70% of human genes have at least one obvious zebrafish orthologue[4] . This has also become an attractive model because of the possibility of performing small-scale, high-throughput analyses[5]**.**

Not only are zebrafish ideal for a wide range of toxicological studies, but they are also an effective model to use in undergraduate laboratories. Adult zebrafish can easily be kept in institutions with space constraints, and the breeding of zebrafish can be done quite effortlessly without much prior experience. Using zebrafish as a model in a toxicology lab introduces students to the use of vertebrate organisms, and it enables them to design, carry out and analyze experiments. The design of this lab is suitable for examining the developmental, mortality, and behavioral effects of common environmental toxicants. This lab can be modified depending on the capabilities of the institution, and the toxicants can be tailored to both the need and interests of the students.

This laboratory is intended for an upper-level undergraduate toxicology course that meets for three hours each week. In addition to the normal lab hours, students are required to come in outside of class time to periodically monitor development. The students are responsible for all of the exposures for their independent projects. In the interest of time and conservation of materials, the experiment is best performed in groups of 2 or 4 students so that the work can be divided. The objectives of this lab are for students to become familiar with zebrafish as a toxicological model, to become skilled in the formulation of independent experiments, and to understand the use of statistics for the analysis of data. The use of independent experiments has been shown to be an effective teaching tool to reinforce course concepts and enable students to explore their own interests while developing important scientific skills[6].

## Equipment

To conduct this series of experiments there must be access to early stage (6 hour post fertilization-24 hour post fertilization) zebrafish embryos. These can be reared in house[1-3] or purchased from outside vendors. There must also be access to incubators; dissecting microscopes with digital imaging capabilities; and, for behavioral assays, EthoVision (Noldus). Ethovision is a tracking system for automatically recording and quantitating animal activity and movement. If the institution does not have Ethovision, then behavioral measurements can be omitted. Lastly, zebrafish are a vertebrate organism and therefore are subject to Institutional Animal Care and Use Committee (IACUC) approval.

**Experimental Overview**

This lab is divided into three major sections. In the first week, students observe normal development of zebrafish embryos from six hours post fertilization to 7-9 days post hatching. In the second week, zebrafish are exposed to nicotine, retinoic acid, or ethanol and students collect data on overall development, behavior, notochord length, and dry weight. These particular chemicals were chosen because they have been shown to elicit developmental and behavioral changes[7-12]. These parameters are based on capabilities of the institution, ease for undergraduates, and they allow for statistical analysis. Endpoints can be tailored for intuition and level of course. Lastly, students write a proposal for an independent project in which they expose zebrafish embryos to an approved toxicant of their choice and collect data to present to the class. The students are instructed that these experiments must have a measurable hypothesis, measure at least two separate endpoints (i.e. development, length, weight, etc.), and the experiment must examine a dose or time dependent exposure to the chemical. For the designing of projects students are required to search the primary literature and find two-three primary articles to support their hypothesis. Prior to conducting the experiment the students must get approval from the instructor. The use of an independent project is an excellent way for students to understand zebrafish as a toxicological model but it also enables them to further understand the scientific method, searching the primary literature, and the use of various biological tests.

*Normal Developmen*t

Zebrafish embryos are collected at approximately 6 hours post fertilization, and 4-5 embryos are placed in each well of a 96 well plate. The embryos are maintained at 28°C, and every other day, water is changed to insure high water quality. Students begin observing the embryos at 6 hours post fertilization and stage development using a dissecting microscope with digital imaging software. It is expected that the students will come to observe the zebrafish frequently over a period of 7 days. Typically students come by every 6-12 hours within the first 48 hours post fertilization because development happens quickly but following that every 24 hours for observation is satisfactory. The students are introduced to the various stages of development using lecture slides and handouts which illustrate the developmental stages of zebrafish[2, 3, 13]. Beginning on day 4 post fertilization, yolk sac exhaustion occurs, and exogenous feeding begins, which continues for the duration of the experiment (approximately 9 days post fertilization). During this time, zebrafish are fed a diet of *Tetrahymena*, a cultured ciliate[1-3]. In addition to monitoring overall development, students use Ethovision to track behavior for the larvae up until 9 days post fertilization[14-17]. The behavioral assays which the students can monitor can vary from T-mazes, to rate of swimming, and direction, all of which can be tracked and quantitated using Ethovision. Larvae surviving until the end of the experiment, are euthanized in a tricaine methane sulfonate solution (MS-222, 0.02% solution) and fixed in 4% paraformaldehyde and transferred to 70% ethanol for further analysis. Preserved larvae are measured for notochord length and dry weight. Notochord length is measured using an ocular micrometer, and qualitative differences in appearance (organ structures present, amount of pigmentation, etc.) are also noted. To determine dry weight, larvae are dried at 60°C for 48 hours, and 5-6 are weighed using a microbalance to determine an average weight[7].

*Toxicant exposure*

Zebrafish embryos are collected and 4-5 embryos were placed in each well of a 24 well plate. The embryos were exposed to toxicants by adding the toxicants to the water. Controls were exposed to water alone. The toxicants used have been documented to induced developmental and behaviorally changes: all-trans retinoic acid (0.01 nM, 0.1 nM, 1 nM, 10 nM)[8, 12, 13, 16], ethanol (1%, 2%, 3%)[10, 11], or nicotine (10, 20, 40 mM)[7, 9]. Over a period of one week, students observe behavior and development, and they collect data on mortality, notochord length, and dry weight to determine if exposure results in variation from the control. The students are also able to calculate the $LC_{50}$ for each of the toxicants, and the data is quantitatively evaluated for statistically significant differences between treatments using a student's t-test. Variations on the experiment can be performed by varying exposure time or the number of replicates, exposing embryos at a different developmental stage, or using different toxicants (arsenic, nocodazole, aphidicolin, paclitaxel, caffeine, lithium chloride, or doxorubicin)[5, 16-19].

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*Independent project*

The last part of the lab, students write a research proposal for an independent experiment of their choice, carry out the experiment, analyze the data, and present their findings. The design of this enables students the freedom to pick their toxicants and to carry out the experiment independently. The students are instructed that these experiments must have a measurable hypothesis, measure at least two separate endpoints (i.e. development, length, weight, etc.), and the experiment must examine a dose or time dependent exposure to the chemical. For the designing of projects students are required to search the primary literature and find two-three primary articles to support their hypothesis. Prior to conducting the experiment the students must get approval from the instructor. Some examples of toxicants that students have investigated include ethidium bromide (1nM, 10 nM, 50 nM, 100 nM), caffeine (0.1 μM, 1 μM, 10 μM, and 20 μM), and lithium chloride (200 mM-10 minutes, 1 hour, 24 hours). All of the toxicants must be approved by the instructor and easily obtained. In order to evaluate the effectiveness of the toxicant, students are required to run multiple tests and conduct statistical analysis of the data. It is expected that students will have prior knowledge of basic statistical analysis including student t-test; however, basic handouts are also provided. Students present their findings in a poster presentation.

*Adult and larval zebrafish maintenance*

Adult zebrafish were purchased from Doctors Foster and Smith and maintained on an ambient photoperiod at 28°C, and fed daily. To collect eggs from adults, a shallow 10x20 cm Tupperware container filled with marbles was placed in the tank with the adults before dark. The following morning fertilized eggs (early stage embryos) were collected by removing the marbles and siphoning out the water in the Tupperware after it was removed from the tank. Fertilized eggs were separated into a 24 well plate with 3-4 embryo/well. Plates were covered an incubated at 28 °C.

## Hazards

Although all of the chemicals in this lab are used in modest volumes and dilute concentrations, care must be maintained. All-trans Retinoic acid is harmful if swallowed and may be damaging to fertility or an unborn child[20]. Paraformaldehyde and tricaine mesylate irritate when ingested or inhaled, with the former also being toxic through absorption[21, 22] . Students should work in a fume hood, and they should wear gloves and goggles while handling all solutions. For the independent projects students must research the hazards of the chemicals and follow proper safety procedures. All of the toxicants used must be approved by the instructor.

## Results

The represented results were collected by students using this laboratory procedure. Their results indicated that 9 days post fertilization (dpf) retinoic acid altered overall growth and survival but had little effect on notochord length (Figure 1). At 6 hours post fertilization (hpf) the exposed and unexposed embryos all appeared to be close to the 1000 cells stage[1-3]. At 36 hpf the control embryos were in the prim 5 stage where a beating heart could be seen. Exposure to 1 nM retinoic acid resulted in embryos at the 20 somite state at 36 hpf, but the heart and optic cup were not visible. The embryos exposed to 10 nM retinoic acid were arrested at approximately the high cell stage. At 48 hpf less than 20% exposed to 10 nM proceeded past the 80% epiboly stage, while 1 nM resulted in the embryos in the prim 11 stage with few that had escaped the yolk sac. The controls hatch, were swimming rapidly throughout the wells, and appeared to be developing normally.

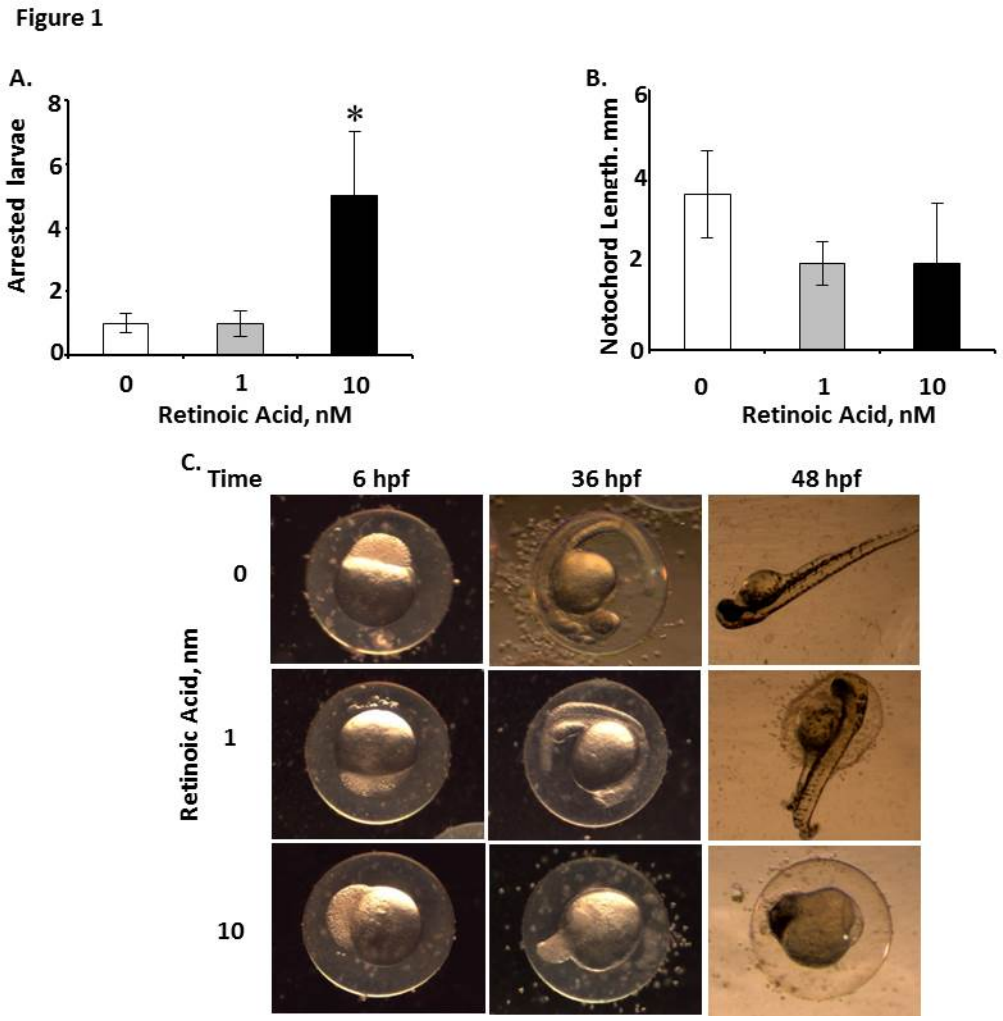


**Figure 1. all-trans Retinoic acid affects survival and overall development of zebrafish.** Zebrafish embryos were exposed to retinoic acid at 6 hours post fertilization (hpf) for 1 hour. Number of arrested larvae and notochord length were determined 9 days post fertilization. (A) Number of surviving larvae following exposure to retinoic acid (B) Notochord length as measured by a micrometer (C) Images were obtained using a Nikon dissecting microscope and imaging software. Data are means ± standard deviation (SD) from eight to ten different larvae. Statistics: * - $p<0.05$.

The results of the experiment are highly dependent on the stage at which the embryos are exposed to the toxicants because some toxicants are timing specific. If it is necessary to purchase embryos, it is typically more difficult to get early staged embryos, and the choice of toxicants may need to be adjusted. The rearing of zebrafish in house is relatively simple, and enough embryos can be produced for a class of 16-20 students using two 10-gallon tanks with approximately 15 fish (male and female) in each tank[2]. This lab can be extended by having the students spend a few weeks learning how to rear and stage zebrafish prior to exposure to toxicants.

## Summary and Conclusions

This experiment has been used in an introductory toxicology course, allowing students to gain hands-on experience with zebrafish as a toxicological model. At the same time, students learned about designing and carrying out an experiment and data analysis. The ability for students to design their own experiment and use toxicants that are available at home piqued their interest about the field of toxicology. Overall, independent projects have been shown to be an effective teaching model.

## Assessment

For this lab the students are assessed on a variety of assignments. When examining normal development students are required to construct a figure depicting the different stages of development. Following toxicant exposure, each student creates a figure comparing control to two different toxicant conditions. Additionally, they are must create two separate figures examining notochord length, dry weight, or mortality. Each of these must include statistical analysis of the data. For the independent project students are assessed on searching and reading the primary literature, the research proposal that they design, collection of data and analysis. Lastly, each group of students presents their final results to the class in power point presentation and they are evaluated by their peers and the instructor.

## Notes

The author declares no competing financial interest.

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