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Using Zebrafish to Explore Development in a Large Introductory Biology Laboratory Course

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Zebrafish are excellent animal models for undergraduate teaching labs. In addition to being inexpensive, zebrafish produce hundreds of transparent, rapidly developing embryos. A four-week module using zebrafish was developed for use in an Introductory Biology laboratory course. Students were given information about six different chemicals with either known or suspected teratogenic effects in humans: lithium, valproic acid, retinoic acid, ethanol, thalidomide and permethrin. Students selected a chemical of interest and designed an experiment to test the effect of their chosen chemical on zebrafish development. After fixing and storing treated zebrafish, students examined their samples for developmental abnormalities. Next, students worked together to compile their data, explore connections of their findings to human health, and prepare a 15 minute presentation to be given during the last week of the module. In this lab, students were able to visualize the consequences of altering early development and make connections to human developmental abnormalities.

Keywords: zebrafish, development, teratogen, inquiry-based

Introduction

Zebrafish have been recognized for many years as powerful animal models for developmental and medical research. In recent years, they have become more popular for use in educational spaces (Felzien, 2016; Fields et al., 2009). They can be raised without expensive commercial equipment and can be bred in the lab with simple techniques. Females can be bred weekly and under proper care, can produce hundreds of embryos. These embryos are easy to observe and develop quickly into hatching larvae after only 48 hours. These features make zebrafish attractive model systems to engage undergraduates in an inquiry-based laboratory setting.

This lab was designed and implemented in an Introductory Biology lab geared mainly towards Biology majors with an enrollment of ~375 mostly freshman and sophomore students. Students were required to have completed the first semester of Introductory Biology which covered ecology and evolution. In the second semester of this course, this lab was positioned at the end of the term after students had covered basic laboratory skills like pipetting and microscope use as well as the concepts of cell biology and genetics. This lab was developed with a number of learning objectives in mind. After completing this lab, the student should understand: the utility of animal models in biology research, how to design a simple experiment to test the effects of chemicals on vertebrate development, how to collect and analyze phenotypic data, how to organize data and present experimental results to the class, and the connection between the observed developmental defects and potential parallels to impaired human development.

Each section of 20-24 students was divided into 4-6 groups of 4-6 students each. Sections (16 in total) were held concurrently Monday through Thursday. Labs met once a week for a maximum of 2 hours 50 minutes although some weeks did not require the full time to complete. The lab as described here covered four weeks: Week 1- experimental design and setup, Week 2- phenotypic examination, Week 3- data collection and analysis, Week 4- class presentations.

Implementation does require the use of live animals and access to or set up of a breeding zebrafish colony. For the purposes of this multi-week lab, a small zebrafish colony was established using two 10-gallon aquariums housing 20 male and 20 female zebrafish. With this modest setup, nearly two thousand embryos were generated during the experimental week; the amount a lab of this size required. While not technically difficult, generation of sufficient embryos for the students required a rotating breeding schedule using fish that had been carefully maintained for at least one month ahead of time. This does require a significant time commitment and attention to detail.

Zebrafish as vertebrate animals, fall into many institution's lists of regulated model systems and may require approval from Institutional Animal Care and Use Committees (IACUC) depending on institutional policies. All work described in this manuscript was conducted under the approval of the William and Mary IACUC.

Student Outline
Week 1: Experimental Design and Embryo Treatment

Objectives

Examine zebrafish at various stages of development
Select a chemical and design an experiment to test the effect of this chemical on zebrafish development
Treat zebrafish embryos with your chemical of choice using your experimental plan

Introduction

During this week's exercises, you will be working with living embryos. Our animal model is the zebrafish (*Danio rerio*). Zebrafish are terrific animal models and have been used for many years to study developmental biology. While fish don't look much like humans, the processes that control how the zebrafish develops are the very similar as those in higher mammals. The nice thing about zebrafish is that they are MUCH easier to work with than traditional mammalian models like mice and rats. The embryos produced by these fish are transparent, develop quickly outside of the mother and are easy to visualize and handle. These are just some of the features that make this model system perfect for learning about developmental processes.

When working with vertebrate animals, we must adhere to policies set by the federal government and William and Mary. These policies mandate that the investigator follow specific guidelines to ensure that animals are not subjected to painful procedures, the number of animals that will be used is not excessive, and that the data obtained using these animals will be beneficial to science or education. The investigator must be knowledgeable about the animal model and must undergo special training on how to recognize and minimize pain and suffering. The protocol that you will be following was approved by the Institutional Animal Care and Use Committee (IACUC) which is a group of faculty including veterinarians and experts in laboratory animal care here at William and Mary. In order to get permission to use zebrafish in this lab, your Coordinator needed to obtain approval for all experimental procedures involving these animals.

You will perform your experimental manipulation on zebrafish embryos during the early developmental stages. You will only be exposing embryos to chemicals during the first 3 days (or less) of development. During this time, the embryonic nervous system is not yet fully formed and the embryo cannot feel pain. After 3 days, your embryos will be transferred to normal water and allowed to develop until they reach 5 days of age. Zebrafish at 5 days of age do not yet have fully developed mouths or digestive tracts and remain dependent on the yolk for nutrition. When your embryos reach 5 days of age (now called larvae) they will be humanly euthanized and preserved for your examination. Should embryos reach a point prior to 5 days where they appear to be near death, they will be euthanized. Remember that we are using these animals to gain an understanding of how these chemicals affect humans. We certainly don't want to experiment on humans to find out if a drug or chemical causes developmental problems, so we must use animal models. Should you feel uncomfortable for ANY reason with working on these zebrafish embryos, please let your Coordinator know and an alternate assignment will be made available.

Last week you will begin your study of development. Your first exercise will be to observe the process of development in our chosen model organism, zebrafish. The processes that control how the zebrafish develops have been studied for many years and much is known about the importance of these pathways for proper development. After you become familiar with observing and handling embryos, you will design an experiment to test the effect of a chemical/drug/toxin on zebrafish embryonic development. The chemicals you will be testing were not picked randomly. These are all chemicals that may impact human fetal development.

You will work in groups of four for this and the rest of the labs in this module with the grand finale being a 15-minute group presentation of your experiment. Select a group name or number to identify your plates/embryos. Next week you will look at the results of your experiment and perform a staining procedure to examine development of the cartilage in treated fish. The following week you will compile your data, perform your analyses and work to prepare your slides. Our last lab week will be where you present your work to your class.

Your mission this week is to (1) become familiar with normal development in zebrafish (2) pick your chemical of choice, (3) design your experiment, (4) treat your embryos following your experimental plan.

The drugs/chemicals you will select from cover a wide range of mechanisms of action and potency. Most are known teratogens, chemicals known to cause abnormal development. You will have a stock solution of the chemical you choose to make your treatment doses. Remember that a stock is just a concentrated solution that is used to make dilutions.

Safety

Please wear gloves and lab coats for all portions of this experiment. You will be working with very diluted chemicals but some are still toxic. If you spill any of the chemical, please let your TA know immediately. Discard all tips in the bins provided on each bench.

Methods and Data Collection

Part 1: Observing Normal Development

Now that you have mastered basic microscopy skills, you will be making observations on embryonic samples. You will also be practicing the fine motor skills required to handle embryos without smashing them. The developmental stages available for viewing may vary depending on the breeding success of the fish. Work in your group and use your "Experimental Plan Worksheet."

1. Select one of the dishes of embryos. Remember, availability will probably vary depending on the day and breeding success of our animals. Make sure to note the age of embryo or time of fertilization written on the top of the dish you selected.
2. Using a plastic transfer pipette, carefully suck up 2 embryos and place them in a small petri dish. It can be helpful to place the dish of embryos either on the white bench liner or on the black lab bench in order to see them with your unaided eye.
3. Fill the petri dish with the provided solution just so the bottom is covered and your embryos are free-floating in the dish.
4. Return to your work station and using the microscopes at your disposal, observe your embryos trying out the different scopes and the different magnifications available on each.
5. Find what you consider to be the best magnification for the embryo you are observing and draw what you see on the worksheet provided. If you are looking at live embryos, determine the age of the embryos by looking at the current time and subtracting the time of fertilization that you noted in step 1. Record this on your worksheet. Make note of the total magnification you are using and estimate the size of the embryo using the skills you learned in Exercise 4 last week.
6. Now use the staging diagram on the top of your worksheet to identify the stage of the embryo you are observing and record this on your worksheet.
7. Check the other embryo you selected and see if it is at a similar stage. It might help to gently push the embryos you have already examined to the side of the dish using the embryo loop provided. Once you have finished with your embryos, return them to the communal dish.
8. Now repeat this process for any other age of embryo that is available to your lab. Make at least 3 observations.

Part 2: Chemical Selection

Work as a team to pick one of the following chemicals to use in your experiment. Record your selection on your "Experimental plan worksheet".

Lithium- We will be using the chloride salt form to deliver lithium to the embryos. Lithium is commonly prescribed for the treatment of bipolar disorder. While those receiving this treatment are most likely adults, exposure of infants or children to this chemical may occur via pregnancy, nursing, or accidental poising. Lithium is a known teratogen.

Stock concentration: 5 M
Recommended dose range: 0.15-0.3 M
Recommended dosage time: 30 minutes then rinse

Ethanol- This drug is perhaps the most well used in human history. Ethanol is found in alcoholic drinks and is a known teratogen. Exposure to developing humans is most likely to occur via pregnancy and results in Fetal Alcohol Syndrome (FAS).

Stock concentration: 100%
Recommended dose range: 1- 3%
Recommended dosage time: 24 hours then rinse

Retinoic acid (RA)- Retinoic acid is a natural derivative of Vitamin A and is required for proper development. However, excessive retinoic acid is known to lead to developmental abnormalities in several animal models as well as humans. Retinoic acid is often used to treat severe skin conditions. The FDA has now classified RA as Category X meaning it should not be used in pregnant women.

Stock concentration: 0.1 mM
Recommended dose range: 0.2-1 µM
Recommended dosage time: 1 hour then rinse

Thalidomide- This drug was used in the 1950's in West Germany as a treatment for morning sickness. It was later discovered to cause birth defects and death in infants whose mothers received it. It is no longer recommended for use in pregnant women.

Stock concentration: 400 mM

Recommended dose range: 0.1-0.5 mM
Recommended dosage time: 72 hours

Permethrin- This chemical is a commonly used pesticide. In fact, you used a very diluted form of this last semester to prevent tick bites. Currently this compound is not listed as teratogenic but some studies have suggested that it does indeed cause developmental abnormalities.

Stock concentration: 100 mg/L
Recommended dose range: 100-300 ug/L
Recommended dosage time: 72 hours

Valproic acid (VPA)- Valproic acid, also called Valproate, is the most commonly prescribed medication for the treatment of epilepsy. This drug is now a known teratogen and is no longer prescribed to pregnant women.

Stock concentration: 100 mM
Recommended dose range: 0.1-0.6 mM
Recommended dosage time: 72 hours

Part 3: Experimental Design

You will now design your experiment. Each group will have a 3 or 4 small petri dishes depending on how many embryos are available for your section. If there are enough embryos, you will include a control in your treatment plan. Your three dishes will be kept together in a square dish.

1. Before you begin your experiment, take the time to record the following information in your lab notebook:
    a. When you examine your embryos next week, you will be looking for developmental abnormalities. What is the null hypothesis?
    b. What is the alternative hypothesis?
    c. Which dose do you expect to have the greatest effect?
    d. Death is a kind of extreme phenotype. You will be receiving survival data for your embryos. Thinking just about the survival data set, what is the null hypothesis? What is the alternative hypothesis?
2. Look at the recommended dose range in the information above for your selected chemical.
3. Working as a team, select three dosages (concentrations) to treat your embryos. Pick a dose close to or at the low range, pick something in the middle and pick something at the high end. Record your choices on the dosage table on your worksheet.
4. Now you need to calculate how much of the stock chemical you will need to add to achieve your selected doses. You will be treating the embryos in 5 mL of embryo media. Use the formula you practiced in the last module ($C_1V_1=C_2V_2$) to calculate your amounts and record your values in the Dosage table on the worksheet. You should work together to double-check all your calculations.
5. Now fill in the Plate Layout portion of the worksheet with where you will place each dose of your chemical. Remember to leave one well for the control which will receive no drug. You will only be using 4 of the 6 wells so draw an "X" through the wells that will not be used.
6. Show the worksheet to your TA for final approval before beginning the next part of the experiment.

Part 4: Embryo Treatment

Once your TA has approved your plan, begin the treatment process.

1. Obtain 3 or 4 tubes, 3 or 4 small dishes, and one square dish. Label the tubes with your chosen dosages.
2. On the top of the square dish, write your group name, today's date, your lab section # and drug of choice.
3. On the top of each small petri dish, write the concentration for that plate and the drug you chose to work with.
4. Pipet 5 mL of embryo media into each of your tubes. Using your table, remove the amount of chemical you will be adding. For example, if you determined that you need to add 12.5 µL of chemical to achieve your desired concentration, remove 12.5 µL of embryo media from the appropriate tube. The point of doing this is so that your final 5 mL volume doesn't change when you add your chemical. Do this for each of your tubes.
5. Now add your calculated amount of stock chemical to each tube according to your table. Stock chemicals can be found on the middle bench.
6. Cap each tube and mix gently by inversion.
7. Obtain a petri dish containing your embryos. The exact number will vary depending on the breeding success of the fish.

8. Using a plastic transfer pipette, divide your embryos among your 3 or 4 dishes. Each of you should transfer embryos into one of the small dishes if possible. Record the number of embryos you put in each treatment dish on your worksheet.
9. Now each one of your group members should take one of the small dishes. Your next goal is to remove the majority of the embryo media without removing the embryos! CAREFULLY and SLOWLY tip the small treatment dish so the embryos gather at the bottom edge. Using a P1000 micropipette set to 1000 uL, pull the liquid out slowly. The best way to do this without getting embryos stuck is to try to stay at the top edge of the liquid. You will probably need to make several pulls to remove the majority of the liquid.
10. Now pour the contents of each of your tubes into the appropriate dishes and swirl gently to make sure the embryos are free of the bottom of the dish. Record the time on your worksheet and on the top of your square dish.
11. Using your microscopes, determine the stage of the embryos at the time of treatment. Compare what you see to the staging diagrams available in the lab and on your first worksheet. Record the stage of your embryos on your worksheet along with the fertilization time (written on the petri dish).
12. Place plates in the incubator at 28.5 °C.
13. **IMPORTANT FOR LITHIUM CHLORIDE USERS** if you have chosen to work with Lithium Chloride, you will only be treating for 30 minutes and then rinsing with fresh embryo media. It is very important that you let your TA know when you added the lithium chloride to the well so s/he can let the coordinator know and the rinse can be performed.
14. **IMPORTANT FOR RA USERS** Retinoic Acid requires a 1 hour treatment time. It is important that you make sure you record the time on your plate when you added the chemicals. Let your TA know when you add your drug so the wash can be performed by the coordinator at the appropriate time

2: Phenotypic Data Collection

Objectives

Recognize and describe gross phenotypic defects
Examine cartilage formation using stained larvae

Introduction

Last week you performed your own experiment to test the effect of a chemical on the development of zebrafish. Today you get to examine your results by making extensive phenotypic observations. You will look at your preserved fish as well as fish that have been stained with Alcian blue. Alcian blue stains cartilage in young fish. This is a way to more closely examine developmental abnormalities that may not be apparent with unstained fish.

Your embryos were exposed to the chemical you selected for the amount of time described in the protocol last week. After the exposure window, all embryos were washed and allowed to develop in normal embryo media. The embryos were observed every day and any that had died were removed. Once a phenotype was apparent, the embryos were euthanized using approved a method shown to eliminate pain and suffering. After euthanasia, the embryos were fixed. This process preserves the fish tissue, protecting it from decay which allows you to examine the fish at a later date. We use 4% paraformaldehyde (PFA) for this process. Once the larvae are fixed, they can be stored indefinitely or used in any number of protocols designed to examine tissue structure in more detail. Your embryos were preserved at either 3 or 5 days post fertilization (dpf) depending on the severity of the phenotype. This is noted on each tube.

In lab this week, you will be carefully examining your preserved specimens under the microscope. You will compare what you see in your treatments to those embryos that were not treated (control) to find specific abnormalities. Depending on the day of your lab, you may have had a control group. If not, there will be plenty of fixed control larvae for you to examine. You will be recording your observations of phenotype. Phenotype is an observable physical characteristic. There are many different phenotypes and many degrees of severity you might observe, and all the fish you treated in a single dose may not look exactly the same.

You will start with a detailed examination of control fish. Once you have an idea of what “normal” looks like, you will make observations about the fish you exposed to your chemical of choice. Not every fish within a single dose will look exactly the same. Some fish may look normal while others may have specific phenotypic differences like smaller heads, eyes or shorter bodies. Since we need to have a quantitative way to express how severe the phenotypes might be and a way to compare your treatment doses, you will be coming up with a set of phenotypes that you can classify as Mild, Moderate or Severe. This way, you can classify each of your fish as one of these categories. For example, let’s say you observe that some of your fish have a slight curve to the body, normal heads, normal eyes and heart edema. You might classify those fish as “Mild”. Then you have some fish with a more severely curved body, small head, small eyes and large edema. These you would

call “Moderate”. Then there might be more fish that look really terrible. For example, they may be shortened, have only one eye and massive edema. These you would call “Severe”. You will use these categories and count the number of fish from each dose that fall into your classification schemes. This will be your only opportunity to look at these fish and collect this data. You will also be taking pictures of what you see to include in your presentation at the conclusion of this module.

In addition to the preserved fish, you will be given preserved fish that have been dosed with one concentration of your chemical of interest at a time prior to your lab period. This separate tube of fish was stained with Alcian blue. This stain will color cartilage in the fish blue. Using this method, you will be able to see if there are any changes to the developing head structures in treated fish. Because the full staining protocol requires several days, you will be examining the fish after this procedure has been completed. Normally, you can’t see the cartilage in these small fish, but this stain will enable you to easily make out these structures.

In addition to developmental abnormalities, your chemicals could have caused premature death. You can think of this as a kind of extreme phenotype. As we could not ask all students to come in and check their embryos every day, your coordinator checked all dishes and recorded the number of embryos that were dead each day. These dead embryos were removed from the dish to help keep the water clean. Dead embryos break down over time and the resulting “embryo funk” can negatively impact other embryos in the dish. All that data was recorded and will be given to you today.

The data you collect today will be analyzed next week and will go into the presentation you make to your classmates.

Methods and Data Collection

Part 1: Phenotypic Examination

1. Your TA will give you back your preserved fish and your data on survival that was collected between 1 and 5 days post fertilization (dpf). Keep your survival data in your notebook; you will work on analyzing those data next week.
2. Start with the control. If your group did not have a control, use the control fish in the communal dish in your lab room. Using a glass Pasteur pipette, carefully pipet two control fish into a depression slide and label the slide “Control.”
3. Now look at your Phenotype Worksheet. Work your way down the table filling in a short one or two-word descriptor of the characteristic listed on the left. For example, you might estimate the body length as 2 mm and the body shape as straight. Complete the Control column writing something in every box, even if it seems simple or silly.
4. Try to work as a team for this process. You can use either scope but probably start with the dissecting microscope. You can use the fishing line tool to move the fish around to see all angles. Refer to the images at the end of the protocol for locations of various fish parts.
5. Document your control fish phenotype by taking at least one picture either by holding your cell phone with the camera lens close to but not resting against the eyepiece, or by using the scope at the front of the room.
6. Your treated fish will be in tubes. They may be either 3 or 5 days old. Using a glass Pasteur pipette, carefully pipet the fish from the Low dose into a depression slide. You may have to try a couple of times to get all the fish. Remove excess liquid from the depression of the slide so that you end up with a relatively flat amount of liquid. Label this depression slide “Low.”
7. Now examine these Low dose fish under the microscope. Fill out the Worksheet table column for this dose by comparing what you see to what you saw with the control fish. Use the descriptors in the table above as a starting point but feel free to write anything that fits with what you see. You can alternate back and forth between the Low dose and the Control slide. Keep in mind that all your Low dose fish may not look exactly the same. If this is the case, try count the fish that look similar.
8. Document your Low dose fish phenotype by taking at least one picture either by holding your cell phone with the camera lens close to but not resting against the eyepiece or by using the scope at the front of the room.
9. Now repeat steps 5-7 for your Medium and High doses. Label each slide so you don’t forget which is which.
10. Look at the table you just completed and try to come up with a set of phenotypes that you can classify as Mild, Moderate or Severe. Write a description of what you consider each category to be defined by in your lab notebook. For example, let’s say you sometimes observe fish with a slight curve to the body, normal heads, normal eyes and heart edema. You might classify those fish as “Mild”. Then you have some fish with a more severely curved body, small head, small eyes and large edema. These you would call “Moderate”. Then there might be some fish that look really terrible. For example, they may be shortened, have only one eye and massive edema. These you would call “Severe”. This is just an example. Look at what you observed, and come up with a set of criteria that fit what you see. Don’t just think it terms of the dosages, you may see severe phenotypes in doses other than the high dose.
11. On the Worksheet, record the number of fish that fall into each category by dose. Do your best to categorize each fish but this is a subjective determination. Be as consistent as possible with your classifications. Any normal fish should be noted as well (# of normal fish).
12. When you have finished with your fish, carefully return them to their proper tubes and return any control fish you used to the communal dish. Rinse your depression slides and return them to the boxes.

Part 2: Alcian Blue Cartilage Stain

1. Use a glass Pasteur pipette (you can use the same one as Part 1) to move two Control stained fish to a depression slide. Label this slide "Control."
2. Closely examine the stained fish. You should see something similar to Figure 1 at the end of this protocol. Move the fish around using the blue loop tool to see if you can match your fish to the image shown.
3. Now move two Treated fish to a depression slide. Compare what you see to what you observed in the control fish.
4. Record your observations in your lab notebook. Look for changes in the cartilage of the head and fins. Even if you don't see any differences from the control fish, this still means something! Make sure you write down your observations regardless.
5. Photograph your sample even if you don't see anything obviously different from control.
6. Return the fish to the appropriate tube and clean the depression slides returning them to the box when you are done.

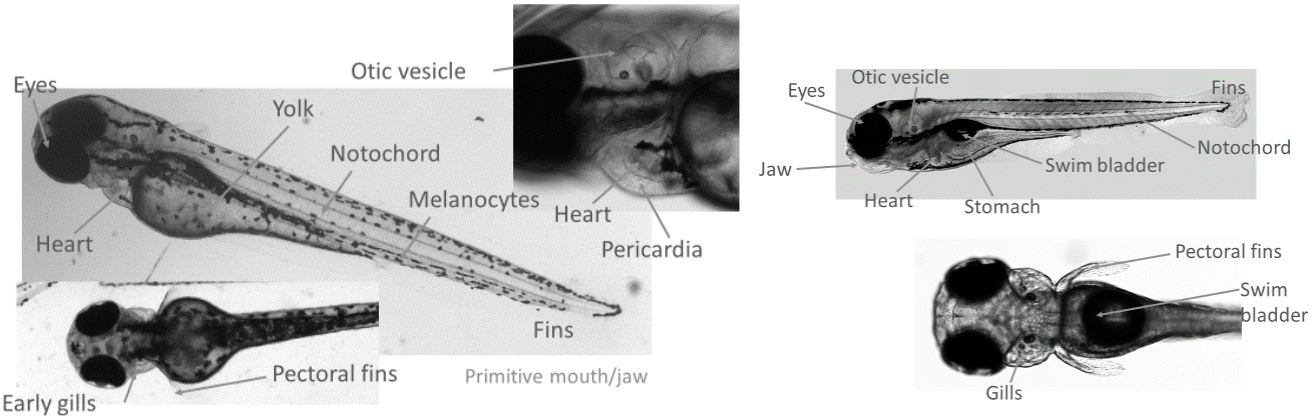

Figure 1. Normal fish at 3 days of age Normal fish at 5 days of age.

Analysis and Interpretation of Experimental Results

Objectives

Use Excel to generate graphs to describe data
Compile and organize images

Introduction

Over the past two weeks, you have conducted an experiment designed to determine the effect of a chemical on the development of zebrafish embryos. This week, you will analyze your data and begin to work on your group presentations. Before you start building your slides, you should work to compile your data. Throughout this week's protocol, you will see questions to answer in your lab notebook. These will help you during your slide preparation. Each person should take part in the data analysis.

Last week you collected data about phenotype. You made observations about the physical appearance of embryos treated with your chemical and hopefully documented these observations. You also examined the cartilage formation in developing fish using the Alcian blue stain. Survival data was collected for you over the course of the week. These data were given to you last week. The first part of this this week's exercises is devoted to analyzing these data and preparing appropriate graphs or tables. Once you have finished preparing these data, you can begin to work on your presentation. Refer to the Group Presentation Information document for details on how you should structure your presentation.

Part 1: Data Analysis-Survival

Remember that death is just a kind of severe phenotype. If your embryos die at a certain dose, it is important data. Toxicity is a very important piece of information to have about any chemical, especially one that will be used to treat a disease.

1. Before you start your data analysis, think about your experiment. Write down the following in your lab notebook:
    a. What is the variable you were testing?
    b. Do you expect to see differences between doses? Which dose do you expect to see the highest/lowest survival?
2. Last week you received data on embryo/larva survival for your experiment. Find this data.

3. Enter this data into an Excel spreadsheet. You can keep the same column and row orientation. Make sure to change your column titles to reflect your selected doses.
4. If your section did not have a control, leave that column out of the analysis.
5. Now make a line graph. Make sure you label your axis on both X and Y.
6. Now answer the following question: Did your drug impact survival?

Part 2: Data Analysis- Phenotypes

1. Next you will want to analyze your phenotypic data. Refer back to your data sheet where you recorded your phenotypes.
2. Before you begin analyzing you data, think back to your experiment and answer the following questions in your notebook:
    a. What is your null hypothesis?
    b. What is your alternative hypothesis?
    c. What do you predict the effect of dose will be on your phenotypes?
3. Look back at your phenotypic observations from last week. Transfer your data on phenotype category to Excel. Keep the same column and row orientation.
4. Now add a graph. Highlight just the first four rows (Total # of fish row is included just for your reference) and then click Insert. Good options for the Chart Type include under Column Chart either Stacked Column, Clustered Column or 100% Stacked Column. Try them all and see which one you like better. Keep in mind that each type of chart is used to show the data in slightly different ways. Stacked and Clustered columns show only the fish that were alive while the 100% stacked column chart turns each category into a proportion of the whole.
5. Looking at your data, do you accept or reject the null hypothesis? Did you see differences in phenotype category between doses?

Use the remaining time this week to work with your group on your presentation. You should use a platform like Google Slides to work together collaboratively. Every member of the group should participate in the preparation and delivery of the presentation.

Week 4: Group Presentations

Group presentations are worth 50 points. Each presentation must be 15 minutes long with every group member contributing equally. Each member of your group will present a portion of the slides. You can divide up the sections among yourselves but remember to work together, not as independent people. Your sections should be cohesive and coordinated. Although each of you will only be presenting one section, you should each be able to step in to any section should the need arise. Be consistent with color schemes, backgrounds and fonts. Your presentation must contain the following sections:

1. Title slide: This should have a title for your project and everyone's names
2. Introduction and Experimental Design (2-3 slides; 3-4 minutes). This section should set up the experiment. Imagine you are talking to someone who has no idea of what you did. Make sure you include the following information:
    a. What was the question you were testing?
    b. Chemical of choice and short background on your chemical
    c. Doses selected
    d. Age of embryos at time of dosage
    e. Number of embryos per dose
    f. Treatment time
3. Results and Analysis (3-4 slides; 3-4 minutes). In this section you need to report your results. Include the following in graph/tables/pictures:
    a. Survival data (did your drug impact survival?)
    b. Phenotypic data (what phenotype did you observe and how many fish per treatment did you see with that phenotype)
    c. Alcian blue results (did you see any differences between treated and control fish using this dye??
    d. State what you conclude from your experiment
4. Outside study (2-3 slides; 3-4 minutes). You will need to find one scientific paper that is relevant to your experiment. It may be a similar study in zebrafish or in another organism. It may be a study in humans. The point is that it should say something about the effect of your chemical on development. In this section, you need to present

some of the findings from that outside paper. Pick the findings that most directly relate to your study. As long as you cite the paper you can take screen shots of the relevant figures to include in your slides. You should include the following:

a. Citation of the paper and rationale for your selection
b. Reason for the study (hypothesis or question they were testing)
c. Experimental design of the study (organism, drug concentration, treatment times)
d. Results (you can include the most relevant figures)
e. State what they concluded from their study

5. Discussion and conclusion (2-3 slides; 3-4 minutes). Here's where you bring it all together. You need to make a connection between the outside study and what you found. You should address the following:
    a. Did your outside study find similar results? If not, why might that be? Was the model organism different? Did they treat at different doses or different developmental stages?
    b. What is the connection to human health? Did your experiment reveal information about the impact of your chemical on developing humans?
    c. What future directions might you envision?

Materials

Materials listed here are needed per group (here 4-6 students per group and 4-6 groups per section): dissecting and compound microscopes, 35 mm petri dishes, plastic dropper pipettes, chemicals for treatment (all purchased from Sigma): lithium chloride, ethanol, retinoic acid, valproic acid, thalidomide, permethrin, micropipettors and appropriate tips, 15 mL conical tubes, thin wire probes (or fishing line taped to regular probes), incubator capable of maintaining 28.5°C, glass Pasteur pipettes, zebrafish embryos, treated zebrafish embryos pre-stained with Alcian Blue

Notes for the Instructor

This lab requires a significant time investment in setup and prep. I have previous experience setting up and maintaining zebrafish but it is possible to setup a colony with no previous experience. There are a number of online resources with valuable information to help: www.zfin.org, www.zebrafish.org are two places to start. I was able to establish my breeding colony of zebrafish using 10-gallon tanks set up with gravel, one air stone, a heater and a power flow filter (available as a kit from Wards/VWR). Tank temperature was maintained at 28-30°C and the fluorescent light hoods were connected to a timer to maintain a 14/10 hour light/dark cycle. Males and females were housed in separate tanks and fed three times daily: morning- Tetra flake food, mid-day- fresh live brine shrimp, afternoon- Tetra flake. Including live brine shrimp is critical for quality egg production. Water quality is also critically important for breeding success. Tank water was made from in-house DI water with the addition of 5.7 mL Instant Ocean solution (40 g/L) per gallon, 0.6 mL sodium bicarbonate (35 g/L) per gallon, and 0.5 mL Stress Coat (VWR) per gallon. Twice weekly, one half of the water in the fish tanks was siphoned out and replaced with fresh tank water. Ammonia levels were monitored twice weekly, and pH and temperature were measured daily. Fish were obtained either from VWR, Carolina Biologicals, or an on-line pet distributor PetSolutions.com.

The day before embryos were needed, another 10-gallon tank was set up with a layer of marbles on the bottom. A piece of clear Plexiglass with holes drilled in it was placed in the middle of the tank dividing it into two halves. Fresh tank water was added and a heater set to maintain 28-30°C. An air stone was added but not filtration as the fish were only be housed here temporarily. Once the tank had reached the proper temperature, 6-8 females and 3-5 males were moved from the housing tanks to each side of the breeding tank. The tank was wrapped in a dark fabric to keep out ambient light, and the fluorescent hood lamp placed on a timer. The next morning before the tank light was scheduled to turn on, the divider separating males and females was removed and the fish were allowed to spawn over the marbles. Once spawning had concluded (typically 1 hour after the light switched on), fish were removed and the marbles were carefully removed from the tank. The eggs could be easily seen at the bottom of the tank and were removed by siphon. Eggs were counted and checked for successful fertilization before being given to each lab section. The number of eggs distributed depended on the breeding success of the fish. Females do best when bred only once a week, so another tank was set up to house those fish who had been bred already, thereby removing them from available fish to breed until the next week.

Six drugs were selected for study. The majority of these drugs are known to cause developmental abnormalities in humans and were presented as such, but without details on what kinds of abnormalities were associated with *in-utero* exposure. A range of concentrations for each drug was provided to students, and students were instructed to select three doses within this range. Embryos were checked each day by the coordinator and death was recorded. Dead embryos were removed daily. If the phenotype was severe, embryos were euthanized and preserved in 4% paraformaldehyde at 3 days post fertilization. Otherwise, embryos were kept until 5 days post fertilization, at which point the coordinator preserved all that were still alive. Preserved embryos were kept at 4°C until the labs met the following week. Prior to the initiation of this lab sequence, the coordinator treated and preserved a subset of embryos with each chemical and performed a staining procedure with Alcian blue to stain cartilage structures. This procedure was too time-consuming to include in the student portion of the lab.

Students were instructed to document their treated embryos using photography (mostly with their cell phones held up to the microscope objectives). They were also given direction on classifying their embryos into categories of severity. These categories were enumerated and plotted in Excel. Survival data was provided for each group (collected by the coordinator for days 1-5).

I heard from many students that this was their favorite lab module. Student engagement seemed high and most enjoyed the hands-on nature of this module. They also expressed satisfaction in having freedom to pick a drug and doses to treat. The particular pathways impacted by each drug were not covered in detail, and that may have hindered student understanding of the mechanism underlying the action of these drugs. The setup and running of this lab module was very time consuming for me as the coordinator. Working with zebrafish is by nature somewhat unpredictable, and despite all my efforts, some days the breeding did not yield sufficient eggs. However, the students could still complete the weekly objective and I would add embryos at a later date when available. There were simply not enough fish to generate a robust number

of embryos per group for statistical analysis. This could be improved by increasing the number of fish available to breed, or by changing the experimental design to only test one concentration but with multiple replicates.

A number of possible modifications could be made to the material to shorten or lengthen the number of weeks covered. Alcian blue staining could be easily omitted as this contributed greatly to the prep time and could not be performed by the students due to time constraints. A written report could be substituted for the oral presentations. If a more in-depth analysis of developmental pathways was desired, this module could be expanded to include drugs targeting specific proteins in pathways of interest. Much of the time and effort of this lab is devoted to zebrafish husbandry. Interested parties may be able to get around this by collaborating with an in-house zebrafish facility or working with other interested faculty to develop a student-run small-scale zebrafish habitat. Overall, this is a logistically challenging module, but I believe that it is worth the effort and could be made better with some small changes.

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