Experimental Embryology: a modern approach to investigating the affects of Teratogens on Early Development.

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Abstract: Hands-on laboratory experiences are a key component to undergraduate education. To fulfill this need, I have developed an experimental embryology lab course that introduces basic problems in developmental biology by direct experimentation. The effects of teratogens on development is an interesting area of study that allows the students to explore normal verses abnormal development. This also provides an opportunity for more advanced students to study how chemical exposure during critical times of development can alter gene expression. The course uses frogs (*Xenopus laevis*), chicks and zebrafish (*Danio rerio*) to demonstrate the usefulness of model systems in research. Both classical embryology and modern molecular manipulation of developing embryos are performed during the lab period, accompanied by an interactive lecture and discussion period. Students learn state of the art techniques by conducting experiments published in current research articles. The course invites both senior undergraduates and graduate students. Many first year graduate students take the class to learn techniques they will use in their research. Undergraduate students also work in the research lab on complementary experiments and help with preparations and observations outside of the class period.

Introduction

This paper describes tested exercises performed in an experimental embryology lab course. The course introduces basic problems in developmental biology by direct experimentation. The affects of teratogens have been an interesting area of study that allows students to explore normal verses abnormal development. This also provides an opportunity for more advanced students to study how chemical exposure during critical times of development can alter gene expression. The course uses frogs *(Xenopus laevis),* chicks and zebrafish *(Danio rerio)* to demonstrate the usefulness of model systems in research. Both classical embryology and modern molecular manipulation of developing embryos are performed during the lab period, accompanied by an interactive lecture and discussion period. Students learn state of the art techniques by conducting experiments published in current research articles. The course invites both senior undergraduates and graduate students. Many

first year graduate students take the class to learn techniques they will use in their research. Undergraduate students also work in the research lab on complementary experiments and help with preparations and observations outside of the class period.

Evaluation in this course is based on weekly lab reports, an oral presentation and a final lab notebook with corrected reports including conclusions and discussion. There is also a take home final exam that poses a question that requires knowledge of the procedures done in class and a familiarity with the literature covered in the lecture and discussion period.

Student Outline

Situation:	Normal verses Abnormal Embryonic Development.
Hypothesis:	If gene expression influences how an animal develops and chemicals can alter gene expression, then exposure to chemicals such as teratogens or hormones should cause an animal to develop differently.
Question:	What affect do chemical exposure (teratogens or hormones) have on Development?
Exposure Experiments:	Thyroxine and Biphenyl-A (BPA)
Techniques:	1) In vitro Fertilization, 2) Embryonic incubations under varying conditions followed by phenotypic analysis, 3) In situ hybridization
Data collection:	1) Observe embryos, 2) Count phenotypes and take measurements, 3) Document development by photos and/or illustrations.
Data analysis:	Comparative analysis of normal verses abnormal phenotypes.
Reporting Research Findings:	1) Collect data in notebooks; 2) write up analysis and conclusions; 3) make Figures and Tables to show results.

Three related lab exercises are described below. By doing related labs student have more than one opportunity to perform techniques, better understand the continuity of doing research and can also provide samples for future labs. Exercises 1 and 2 are suitable for undergraduate students while exercise three may be best suited for graduate students.

In vitro Fertilization (Lab Exercise 1)

Objective:

The objective of this exercise is to perform *in vitro* fertilizations, learn to stage frog embryos according to the normal *Xenopus leavis* development table by Niewkoop and Farber (http://www.xenbase.org/atlas/NF/NF1-10.html), and to learn how to capture and saving images.

Exercise:

Embryos are fertilized according to the protocol below by mixing with fresh sperm collected from dissected testis. Each student gets a dish of eggs and a tube of sperm for the procedure. The healthy embryos are collected and the embryonic stages are identified. Individual dishes will have embryos of the same stage but dishes fertilized at different times will contain a different stage. Images are captured and saved on a computer disc. Students should label figures with stage of development, animal pole, vegetal pole, dorsal side, ventral side and any other obvious structures present at that stage.

Exposure to Teratogens (Lab Exercise 2)

Objective:

The objective of this exercise is to set up experimental conditions for chemical exposure. During this lab the students begin the thyroxine experiment and BPA experiment.

Exercise:

In vitro fertilization is performed as before for stage 6 embryos. This can be done prior to class or by another lab section. Collect healthy embryos at stages for experiments. Expose stage 6 embryos to BPA. Expose stage 52 (hind legs emerging) embryos to Thyroxine according to protocol. Note that these embryos can be from prior weeks when the initial fertilization lab was performed. Alternatively they can be ordered already fertilized.

In situ hybridization (Lab Exercise 3)

Objective part 1:

The objective of this exercise is to make synthetic RNA for in situ hybridization probes (Guille 1999: chapter 5 section 3.1), analyze the morphology of embryos exposed in the previous lab exercise and fix with embryos for *in situ* hybridization in the next lab periods.

Exercise:

DNA plasmids of the genes of interest are linearized with restriction enzyme and the template DNA is cleaned up by phenol extraction. The transcription reactions are set up according to the RNA Probe Protocol to produce RNA labeled with digoxygenin. The quality of the RNA is checked on an agarose gel and quantified for probes.

Objective part 2:

The objective for this exercise is to begin the *in situ* hybridization on embryos fixed in the previous exercises.

Exercise:

The embryos are washed and the hybridization is set up according to *in situ* hybridization protocol (Guille 1999).

Procedures

1. In-vitro Fertilization[1]

Solutions for fertilizations include 1X Ringers (saline), 0.1X Barth's solution plus 1000 µl (1 ml) of penicillin antibiotic added per liter, and cysteine (5 g in 250 ml, pH 7.8). The frogs are primed with 50 units hCG at least 5 days before the frog is needed for fertilization. To induce egg-laying the night before fertilization, you will need1X High Salt Barth's for the frogs to lay their eggs in. Pour 1500 mls in each tank for the two females. Inject frogs with 250-300 µl hCG (500 units) to ovulate. Anesthetize the male in MS-222 for 30 minutes, put on ice, then dissect testis. Leave testis in 4˚ refrigerator overnight and cover with parafilm; label dish.

When doing a fertilization, never pour Barth's or water solution directly onto the frog eggs. Always pour it slowly down the side of the dish. Also, always use purified water. The female frogs will lay their eggs overnight in high salt Barth's solution. Remove the eggs using a 25ml serological pipet with a cut tip (for a larger diameter). Put the eggs into a sterilized (autoclaved) glass petri dish by allowing them to fall to the tip of the pipet, then releasing them into the dish so that there is little Barth's accompanying the eggs. Draw off as much barth as possible utilizing a cut plastic transfer pipet; tilt and rotate the dish to help the Barth's solution separate from the eggs. Cut one testis at a time into 3 or 4 pieces. Mince using forceps and mix in 750µl of Ringers in an Eppendorf tube. Divide the testis solutions among the plates to be fertilized, being sure not to put too much sperm on the eggs (causes polyspermy). One tube usually works on 2-3 plates. Swirl the eggs with a pipet tip and then cover the dish. Record the time of fertilization on the dish and let them sit at room temperature for 5 minutes. Add approximately 100ml of distilled water to each dish, cover, and let sit at room temperature for 20 minutes (make cysteine pH 7.8 at this time). Decant water and add enough cysteine to cover the eggs. Cover the plates and put them on the shaker at speed 60 for about seven minutes, or until the jelly coat is removed. Decant the cysteine and wash three times with distilled water. Wash three times with 0.1X Barth's solution. Incubate the eggs at room temperature in 0.1X Barth's; they should begin cleaving about one hour and twenty minutes after fertilization. Leave the embryos at room temperature for faster cleavage, or put them into the incubator (15- 18 degrees) for much slower development, making sure they have a lot of Barth's solution to avoid evaporation.

2. Exposure to bisphenol-A (BPA)

BPA was dissolved in 100% ethanol and diluted in 10% Steinberg's solution. The final concentration of ethanol in the solution was less than 0.2%. Embryos were exposed to BPA once they had reached stage 6. Control embryos were developed in Steinberg's solution containing ethanol alone. Eggs were placed in 24 well cell culture plates for exposure and growth (Figure 1A.).

3. In Situ hybriziation[2]

Embryos were scored for gross morphological abnormalities and fixed at various stages. Whole mount *in situ* hybridization was done on 0mM, 20µM, and 30µM exposed embryos at stages 19, 23, 33, and 42. Embryos were hybridized with probes for the *Xenopus claudin 3* gene to show changes in expression patterns and used to determine and identify the changes in gene expression in the exposed embryos compared to the controls (Figure 1 B and C).

4. Thyroxine Experiment[3]

Divide the class into four groups; each group needs 2 aquaria with 5L of dechlorinated tap water in each; 5 sheets of millimeter graph paper; 2 finger bowls; and 1 small aquarium net for the tadpoles. Mark the 5L water level on the outside of each aquarium. Label the aquariums:

- Group I- Control
- Group II- 10^{-5} *M* Thyroxine
- Group III- 10^{-6} *M* Thyroxine
- Group IV- 10^{-7} *M* Thyroxine

Use an aquarium net to transfer 5 tadpoles to each aquarium (be sure they are all from the same fertilization). Take out one tadpole from the aquarium and transfer it to a finger bowl with refrigerated or ice water. The cold water will anesthetize the tadpole, slowing it down so that observations can be made. Gradually add ice cubes to prevent shock. Slide a sheet of graph paper under the finger bowl as a way to measure the tadpoles. Or, use a short ruler to measure the tadpole. Using a net, return the tadpole to the aquarium. Add a small amount of food to the aquarium (measure the weight first) and observe how much they can eat in a 24-hour period. L-thyroxine can be purchased in powder form from Sigma. It must be dissolved in a small amount of 95% ethanol and then slowly mixed into water. A stock solution of 0.1M can be made, pH drop wise with 1M NaOH until in solution. Keep your thyroxine stock solution refrigerated and in foil-wrapped tubes or opaque glass bottles. Carefully shake each bottle before taking out the correct amount.

- Group I- Control- no thyroxine
- Group II- 5ml thyroxine stock solution *per aquarium*
- Group III- 0.5ml thyroxine stock solution *per aquarium*
- Group IV- 0.05ml thyroxine stock solution *per aquarium*

Set the aquaria aside and adjust the aerators. Observations must be made next week on the same day. Observations are recorded in student notebooks for the next four weeks. The tadpoles must be fed daily and have their water changed on a daily basis as well. Make sure you add thyroxine to Groups II-IV.

For each group of tadpoles, record the following observations:

- Head width (mm)
- Tail length (mm)
- Total Body Length (mm)
- Appearance of limbs, tail, etc.
- Activity Level
- Deaths, if any

If a tadpole dies, please note when and how it died (do your best to estimate). Record the final metamorphic changes by dissecting the tadpole as soon as possible. Tadpoles may be preserved in 70% ethanol if dissection cannot be performed immediately. At the end of the experiment, surviving animals will be sacrificed and dissected in order to compare metamorphic changes in visceral organs as a result of tadpole exposure to different concentrations of thyroxine.

Figure 1. A) Exposure to teratogen in 24 well plates; B) Control embryo (0mM); C) embryo after exposure to 30 mM BPA. B and C show *in situ* hybridization using a *claudin* 3-digoxginin labeled RNA probe. Transcripts showed the defects in ear pronephric tubules and brachial arches. Black arrows in the right panel indicate the defect in eyes.

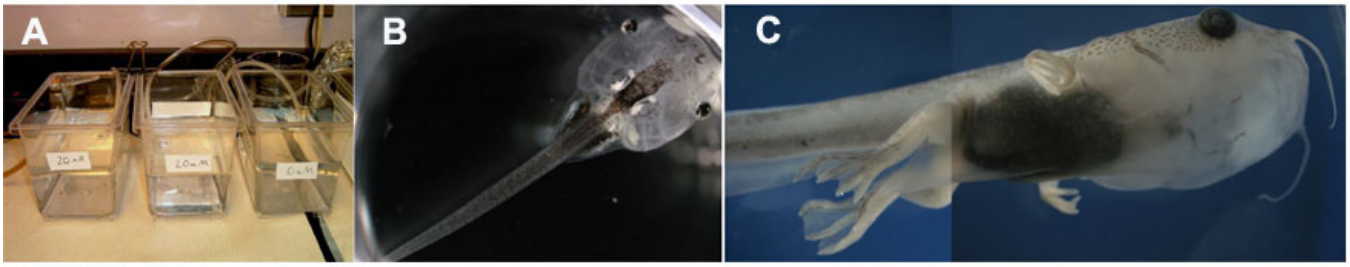

Figure 2. A) Tadpole are treated in small aquarium with thyroxine hormone; B) Premetomorphic tadpole; C) prometamorphic tadpole becomes climatic after three weeks of thyroxine exposure.

Results

Basic experiments were conducted by the undergraduate students. This included *in vitro* fertilization and exposure experiments follow by analysis. In this exercise the students dissect the testis from a frog and learn to successfully produce embryos by *in vitro* fertilization. Tadpole metamorphosis was induced by exposure to thyroxine hormone. Embryos exposed to different levels of thyroxine underwent metamorphosis at different rates. Visible differences were observed in the limbs and tail.

Exposure of embryos to the teratogen bispheol-A (BPA) allowed students to see abnormal development. More advanced experiments were assisted by the graduate students. This included *in situ* hybridization to determined gene expression in the control embryos compared to treated embryos. This exercise included restriction digest of a DNA plasmids, agarose gel analysis, transcription reaction and the hybridization protocol.

Embryonic exposure and Analysis

Exposure to BPA causes morphological defects and altered gene expression of *Xenopus claudin 3* (Figure 1). Treated embryos show a loss of the forebrain region and defects around the eye, brachial arches, otic vesicles (ear) and embryonic kidney.

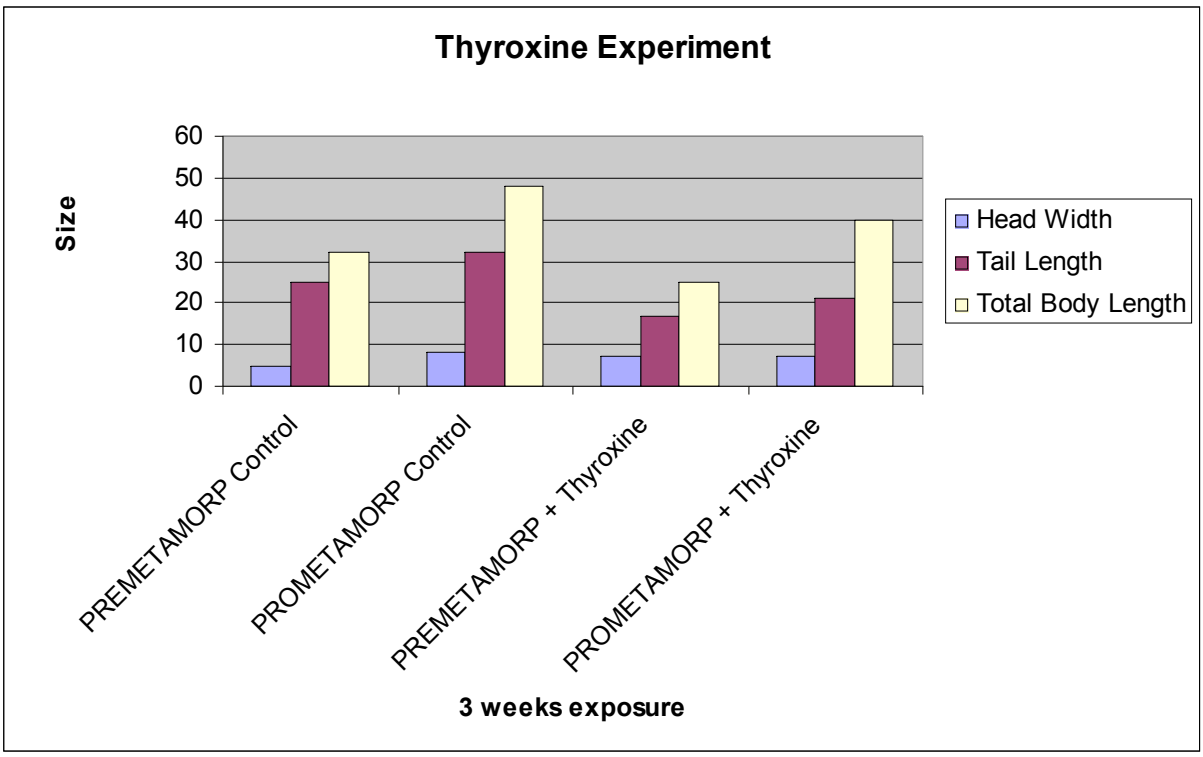

Figure 3. Tadpoles exposed to various concentration of thyroxine hormone show signs of metamorphosis as demonstrated by marked changes in tail length and body size.

Instructor's Notes

Where to buy frogs:

Carolina Biological Supply Company 336-538-6288, Fax 336-538-6337 (Dr. Lisa Darmo)

Nasco 800-558-9595, Fax 414-563-8296

Xenopus I 313- 426- 2083, Fax 734-426-7763 xenopusone@charter.net

Xenopus Express 800-XENOPUS, Fax 888-936-6787 info@xenopus.com

Information on Fish: http://zfin.org/zf_info/zfbook/zfbk.html

Where to find protocols:

- Dr. Cebra-Thomas Experiments in Developmental Biology: http://www.swarthmore.edu/NatSci/sgilber1/DB_lab/DB_protocols.html
- Xenbase http://xtropicalis.cpsc.ucalgary.ca/methods/methods.html

Materials Needed: see website

Links to Author's Websites: http://bbrizuela.pageout.net/

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References

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2. "Molecular Methods in Developmental Biology *Xenopus and Zebrafish"* Edited by Matthew Guille. (1999) Humana Press, Totowa, New Jersey

3. Yolanda P. Cruz "Laboratory Exercises in Developmental Biology" **(**1993) Academic Press, Inc. ISBN 0-12-198390-0-4 San Diego, California

About the Author

Brenda Judge Grubb received a B.S. degree in Biology from East Carolina University, a Ph.D. in Genetics from George Washington University (Washington D.C.) and did post doctoral training at UCLA. She is currently an Assistant Professor of Zoology. She conducts research on embryonic development and teaches courses in Developmental Biology, Experimental Embryology and General Biology. http://bbrizuela.pageout.net/page.dyn/student/course/course_content?course_id=82180