

ECOLOGY and EVOLUTION

Week 2: September 6 – September 9, 2011

There are two parts to this week's lab. Part 1 will be a “downstream” wet lab where we set up an experiment with tadpoles and address our lecture theme of Indirect (Environmental) effects on phenotype. This should take approximately 1.5 hours. In Part 2, we will go a bit “upstream” and introduce you to computer simulation techniques in evolutionary mechanisms and address our lecture theme of Direct (Genetic) effects of phenotype in populations. (Procedure begins on page 3).

LAB II – Part 1. Tadpole morphological plasticity

Before coming to lab, read the lab manual and review the following:

- Tadpoles of the United States and Canada: A Tutorial and Key by Ronald Altig, Roy W. McDiarmid, Kimberly A. Nichols and Paul C. Ustach
<http://www.pwrc.usgs.gov/tadpole/tutorial.htm>
- What is a tadpole's spiracle?
- Today in lab from your observations of live *Bombina* tadpoles, where is its spiracle?

Phenotypic plasticity in tadpoles –

Amphibian embryos and larvae are dynamic developmental systems and are used extensively in ecological and evolutionary studies. Today we will use larvae of the Asian fire-bellied toad, *Bombina orientalis*, to explore a variety of fundamental issues (see especially Lecture 4) in 1) the importance of single and multiple environmental factors in development and evolution and 2) experimental design.

The question - Does a potential environmental stressor (water runoff into breeding pools in an urban setting) vary in effect with a second stressor (the threat of predation)? (Note this sentence could be reversed for emphasis and would then read “Does a predation threat vary in its effect depending on water runoff into amphibian breeding pools?) Inadvertent runoff of common garden plant fertilizers, pesticides herbicides, motor oils pose potential harm to amphibians and potentially contribute to the amphibian species decline crisis. Our Canyon water is precious to us. Is there reason to be concerned that there may be chemicals getting into it that are harmful to amphibian development? In addition, the myriad of potential artificial chemical stressors on amphibians may *synergize* with other factors that are also important. Adult male *Bombina orientalis* have been shown to cannibalize their own species' tadpoles, and other studies of predation effects in different species of frog larvae (as seen on video in lecture) have been well documented. Today we will begin to look at the effects of our Canyon's water and variation in exposure to chemical cues that signal potential predation, both separately and in interaction with each other as we learn how to design a symmetrical, randomized, and replicated, two-factor experiment (See Bio Binder Quantitative Experimental Design F-3 and H-11).

The statistical design of the experiment is formally called a multi-factor, randomized, block design. Specifically, our experiment will be a two-factor (with two levels for one factor and two levels for the other factor), completely crossed, randomized, block design.

		Factor One	
		Level One	Level Two
Factor Two	Level Two		
	Level One		

Three null hypotheses will be tested **simultaneously** in this the simplest and most fundamental of experimental designs that allows for a formal test of **factor interaction**:

- H_0 = There is no significant effect of canyon water presence or absence on size or shape as measured by body length and tail height in *Bombina orientalis* tadpoles after 2 weeks of exposure.
- H_0 – There is no significant effect of potential male cannibal chemical cues presence or absence on size or shape measured by body length and tail height in *Bombina orientalis* tadpoles.
- H_0 – There is no significant effect of the *interaction* of canyon water (+/-) and cannibalism cues (+/-) on the size or shape measured by body length and tail height in *Bombina orientalis* tadpoles.

Setting Up a Multi-factor Experiment –The physical design (see below) is supported by a statistical framework.

Experimental Design has two components: a **statistical design** and a **physical design**. The **statistical design** ensures that proper thought is given to the data matrix that will ultimately be constructed AND that **JMP** statistical analyses can properly test the null hypotheses under examination. The **physical design** dovetails the actual materials, equipment, space, and time constraints with the **statistical design**. Both aspects of experimental design must be tended to with careful thought, or the null hypotheses cannot be objectively tested or false conclusions can be made. This happens all the time because it takes training, practice, and (as with all things) using your intuition helps.

We will design this experiment for you. It is your job to 1) understand the design, 2) execute the experiment, 3) perform the statistical analyses of the data, and 4) write a report.

The statistical design - Two factors crossed with two levels of one factor and two levels of the other factor yields 4 unique treatments. This will be repeated as a whole **block** during each of the lab sections on each of the days (2 rooms x 5 days = 10 blocks in total). The goal is for each student to monitor **two** tadpoles through the experiment. There are approximately 24 students in each section. That means in each class we are designing for 48 tadpoles to be divided **symmetrically** into each of the treatments. Since there are **four** unique treatments there will be 12 tadpoles in each treatment. The actual sample sizes in the following statistical design chart for your particular section should be filled in by you and modified over the next 2 weeks. What might cause the sample sizes at the end of the experiment to be different from what we plan? Is this OK? **Symmetry** is the key element here that protects the fundamental statistical structure. The best we can do, because of space and time constraints is $12 + 12 + 12 + 12 = 48$, and then take care of our tadpoles as best we can.

		Water Quality		
		Canyon Water	Lab Water	
Predator Cues	Present	N=?	N=?	N=?
	Absent	N=?	N=?	N=?
		N=?	N=?	Total N=?

Figure 1. Statistical Design. A two by two matrix showing all 4 possible combinations of the two treatments: Canyon Water vs. Laboratory Water and Predation cues present or Predation cues absent. Consider that this is a picture for one laboratory section. There are 10 sections all together (two on each day).

The Physical Design will only make sense after the statistical design (see above) has been put in place.

Procedure – Wash your hands with soap and rinse thoroughly to protect the tadpole from any contaminants on your fingers such as hand lotions, antiseptics, perfumes, soaps, nicotine, etc.

1. Obtain **TWO** HDPE (high density polyethylene) containers with lids. This will be what your tadpoles live in for the next 2 weeks.
2. Carey or Ned will assign each of you a particular treatment via a randomization process. (**Randomization** is a technical term here that you should come to understand as we proceed.)

Each of you will get two tadpoles, and one will live in Canyon water (CW) and the other will live in Lab water (LW).

You will get one random number defining your treatment of Predation cues added (Pred) or No Predation cues added (NoPred).

You will have one tadpole in two of the four possible treatments on the lab bench in two different locations on the lab bench.

The label should be exactly in the following form:

Day of week, Your Full Name, CW or LW, Pred or NoPred, grid # (assigned in step 14)

Depending on the first random number assigned to you:

Canyon Water:

1->12 = Predator cue added = Yellow tape

13 -> 24 = No predator cue added = Pink tape

Lab Water:

1->12 = Predator cue added = Green tape

13 -> 24 = No predator cue added = Red tape

3. The two containers should be carefully labeled. Label with a Black Sharpie on specific color lab tape on two sides AND the lid. A demo will be on display.

4. Using a beaker, add 200 ml of specially prepared laboratory water (LW) (from the 50-gallon container in the middle room between the 2 labs) to the plastic container.

This water (i.e. 20% modified Holtfreter's solution) has the following recipe:

NaCl 136.43 g

KCl 1.95 g

CaCl₂ 3.90 g

MgSO₄*7H₂O 7.80 g

NaHCO₃ 0.80 g

Water added to 50 gallons total volume.

Use 200ml of Canyon water (CW), also in the middle room, for your other tadpole.

5. Use clean forceps and add one penny-sized piece of **single-layer** boiled spinach to the container (approximately 2.0 cm diameter). **Do not overfeed**, as too much spinach now will require more frequent water changes later.

6. When you are sure that the amount of spinach is right, add one (1) volume of solution, depending on your treatment, using a **micropipette** (see Bio Binder Micropipettes O-1). This will be demonstrated for you.

Predator Cue Water = 1000µl DI prepared predator cue water

or

No Predator Cue: = 1000µl DI water

7. Get one clean plastic Petri dish lid and a plastic mm ruler.

8. Get comfortable taking accurate measurements by looking at the ruler and a coin under your dissecting microscope. To the nearest tenth of a mm, how large is your coin?

9. Get a practice tadpole from the practice tadpole tank at the back of the room and look at it under the scope. Look for the beating heart and blood cells moving. Where is the spiracle? What other parts can you list?

Take practice measurements of body length (not including tail length) and tail height.

Return the practice tadpole to the practice tadpole tank when you are through with it.

10. Find Bob, and he will give you two tadpoles and show you how to handle them. This will be done one student at a time, so be patient.

11. Bring the experimental tadpoles to your desk, and gently put the plastic Petri dish and the centered tadpole on top of the mm ruler.

a) Let the tadpole calm down by not touching it.

b) Also without touching the tadpole, wick off as much excess water as you can from the Petri dish with a KimWipe (in the green box in your plastic drawer).

c) Gently shake the Petri dish side to side to slide the tadpole into a “lie-flat” body position.

d) Move the ruler under the dish to measure.

e) Make an estimate to the nearest 0.1 mm (it will be rough) of the **body length (line a, does not include the tail)** and **maximum tail height (line d)** of the tadpole just as you did last week with **ImageJ** (Fig. 2).

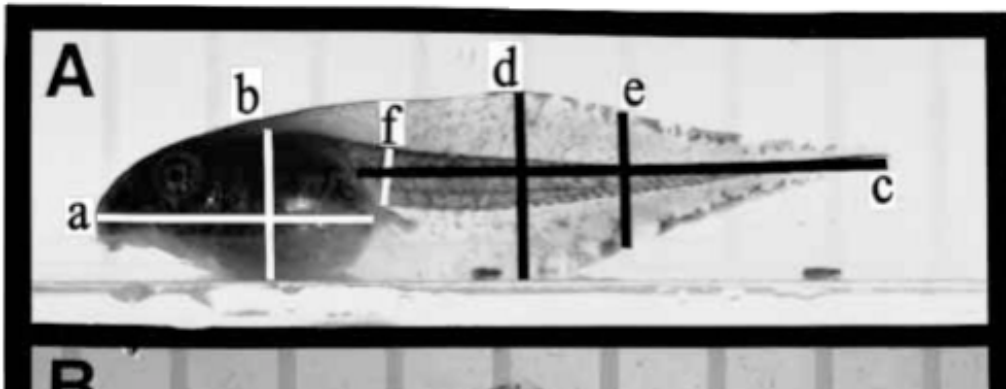


Figure 2. Tadpole body length = line a, maximum tail height = line d

Photo from: Van Buskirk and McCollum, 2000

12. Be sure to write these numbers down in your lab notebook along with whether it is the **CW** tadpole or the **LW** tadpole and **Pred** or **NoPred** cues are added, along with other important details (e.g., time, treatment, grid location - see below).

13. Once you are satisfied with your six measurements (three on each tadpole), **gently** “pour” the tadpole into the **CW** or **LW** container. Place the cover loosely on top without sealing. We want to minimize evaporation, but not seal them in too tightly and risk spills when opening.

14. Get a location for your tadpoles assigned by Carey or Ned. Write the location number on the lids and side labels. Put your tadpoles and container into its proper location in the grids on the lab bench and record its location in your notebook.

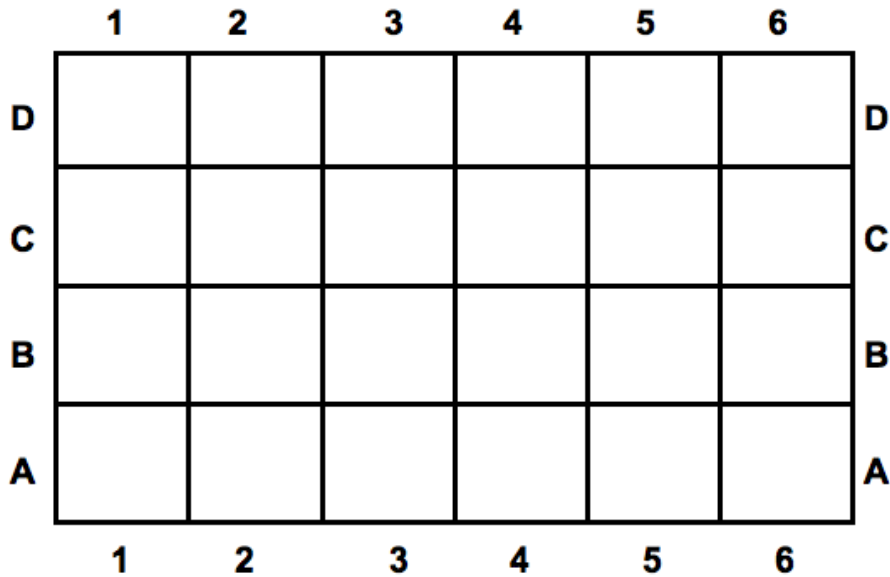


Figure 3. The 4 x 6 class grids are officially called randomized blocks. We have randomized both water source treatment and predation cue treatment in each block. We will have 2 randomized blocks in the experiment in each class, at two different locations on the bench. Consider that 24 tadpoles per block x 2 blocks per class x 2 classes per day x 5 days = 480 tadpoles. Why is this symmetry important? Why will we not be able to maintain it for very long?

An important note about randomization: The **random** positioning of your tadpole in these grids is the second **randomization** we have used in this experiment.

- 1) We **randomized** the treatment that you are responsible for.
- 2) We **randomized** the location of your tadpole in the grids.

We did each of these **randomizations** with computer-generated random numbers,

There are many ways to generate **random numbers**. There are also many ways to incorrectly generate what appears to be a random pattern. Ask your instructors for more information about how to **randomize**.

See next page for data entry instructions.

15. Using the FireFox web browser, enter your results in the lab data collector found at <http://collector.reed.edu>. Go to:

- a) Kaplan
- b) Find your lab section: **Tadpole wk2 Carey** or **Tadpole wk2 Ned**
- c) Follow the instructions carefully.
Abbreviations must match the instructions exactly.
- d) Make sure you measured in **mm** (not cm).

A typical tadpole is 8mm body length and 4mm tail height.

If your tadpole is much larger than 10mm, please check that you are measuring **body** length and not **total** length.

- e) Do not enter units next to your numbers.

16. Subsequent care of your tadpoles (5 minutes EVERY DAY for the next two weeks). Make sure to go over this with us before you leave lab today. Bring your id card. The middle door between B5 and B7 has a card reader to let you in.

a) Bring your lab notebook with you when you come to lab for a few minutes each day to check on your tadpole. Record what you did that day: fed, water change, etc.

When you go in to check, here is what to check for and what to do:

- b) Is your tadpole alive? If yes, proceed to step c.

If it's not swimming, touch it with a clean disposable blue pipette tip to see if it was just resting.

If it's really dead, it will start to decay within a few hours. Pour the contents of the container with the tadpole into the dead tadpole container in the hood. We will take care of it. Record the date and time discovered in your lab notebook.

- c) Is the water cloudy? (This means bacteria and the oxygen depletion that they entail.)

If it needs to be changed, proceed to next step. If not, proceed to step e.

- d) Water changing: as needed if water is cloudy, too much food was added.
- i) Get a clean small (40 mm diameter) petri dish and add some **Canyon water** or **Lab water** from the middle room (not DI water).
 - ii) Move your tadpole with a plastic spoon into the petri dish.
 - iii) Carefully pour dirty water down the drain.
 - iv) Rinse tadpole container with DI water (left tall tap at sinks).
 - v) Add 200 ml **Canyon water** or **Lab water**. Add penny-sized piece of boiled spinach before adding 1000 μ l of **predator cue water** or 1000 μ l **DI water** depending on treatment.
 - vi) Move tadpole back into its container.
 - vii) Put the used petri dish in the dishpan by the back sink.
- e) Does the tadpole have food? If not, add another penny-sized piece of boiled spinach using forceps that are on the spinach box in the refrigerator in your laboratory. Please be sure to put the spinach back in the refrigerator.
- f) Replace the cover and put the tadpole back in its assigned grid position on the lab bench. Record what happened in your notebook with time and date.
- g) Come back tomorrow to check both tadpoles again.

During week 4 - Analysis of the class data will be performed in Lab during Week 4. You will be removing your tadpole, taking the same two measurements, entering them into the computer, and analyzing a class dataset. A report will be due the following week.

Literature

- Kaplan, R. H. and P.C. Phillips. 2006. Ecological and developmental context of natural selection: maternal effects and thermally induced plasticity in the frog *Bombina orientalis*. *Evolution* 60:142-156.
- Earl, J.E. & Whiteman, H.H. Evaluation of Phosphate Toxicity in Cope's Gray Treefrog (*Hyla chrysoscelis*) Tadpoles. *Journal of Herpetology* 44, 201-208.
- Relyea, R. A. 2005. The impact of pesticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications* 15:618-627.
- Sih, A., A. M. Bell, and J. L. Kerby. 2004. Two stressors are far deadlier than one. *Trends in Ecology and Evolution* 19:274-276.
- Van Buskirk, J. and S. A. McCollum. 2000. Influence of tail shape on tadpole swimming performance. *Journal of Experimental Biology* 203:2149-2158.

In preparation for next week's lab, before leaving lab today:

GETTING READY FOR NEXT WEEK'S LAB

Sign up with 1 partner for pond and lake sampling sites on the sheet corresponding to your lab day that is posted in the hall outside of Carey's lab (B7). You will be setting one trap at the pond and one trap at the lake.

Exchange cell phone numbers.

Agree on a time (between 3 and 6 pm) to meet at the Physics loading dock on the day before your regular lab day next week. Monday's lab students will set their traps on Sunday, and we will be here to help you from 4-5pm.

Be sure to come to lab lecture on Monday 1:10-2 pm for more details.

Practice putting a trap together and have one of us check your work. Two hooks and two eyes match on one side to make a hinge, and two single eyes match on the other side and are secured by a clip attached to a rope.

When you leave lab today, walk North on the basement level, and turn left to exit and to see the Physics loading dock where you will meet to find hipwaders, walking sticks, and traps next week starting on the day before your regular lab day.

On your regular lab day, please come inside to lab to receive instructions. We will go out together to collect the traps.