Relating Biochemistry to Morphology Using Inquiry-Based Collaborative Student Research in Developmental Biology
Part 1. Experimental Design

Carl S. Hoegler[1] and Charlene F. Blando-Hoegler[2]
Biology Department[1]
Mount Saint Mary College
Newburgh, NY
hoegler@msmc.edu

Pace University [2]
Department of Biology and Health Sciences
Pleasantville, NY
choegler@pace.edu

Abstract

This study proposes a pedagogical design that encourages students to use animal embryogenesis as a tool for relating biochemical changes to morphological events and organ differentiation. It suggests the use of inquiry-based collaboration in the laboratory of an undergraduate Developmental Biology course. Phase I of this inquiry-based collaboration introduces students to amphibian embryo culturing, protein electrophoresis and database searching using prescribed instructor exercises. In Phase II each student team develops a hypothesis and proposal about their research interest dealing with the effects of chemical or physical agents on embryogenesis. After instructor review, teams complete the project and present results. Our results suggest that this project could be implemented. Data would include mortality and morbidity rates as well as elucidating the pattern of the embryonic protein bands. The strategy and ultimate goal of this pedagogy is to build undergraduate student confidence and analytical skills to help understand the relationship between morphology and biochemistry. In addition we think this will motivate students and sustain their interest in biology beyond college.

Introduction

Classroom pedagogy has been revolutionized by contemporary biotechnology. It fosters an understanding of the fundamentals of nucleic acid and protein biochemistry using an array of analytical tools. At the high school level, instructors may purchase commercially available prescribed kits that illustrate basic concepts like DNA fingerprinting and protein separation by AGE (agarose gel electrophoresis) or PAGE (polyacrylamide gel electrophoresis), resp. College level introductory biology courses often include some version of kits that can be modified to reflect the background and interests of professors who are engaged in their own research. Such kits are carefully choreographed, work within prescribed boundaries and explore a single given hypothesis.

On the other hand, by applying collaborative inquiry based learning as a different pedagogy, excitement has been generated in the college general biology (Luckie *et al*., 2004) or in upper level college level physiology courses, (Kolkhorst *et al*.,2001; Hoegler,2005). When incorporated into the laboratory experience, this approach to learning is more open-ended. It challenges students to come up with their own hypotheses. Toward this end we have developed and propose an animal embryo-based set of experiments to complement the previous seed (plant embryo) study in a Developmental Biology course (Blando-Hoegler and Hoegler, 2009). This new proposal suggests the use of *Rana pipiens* as the model system to encourage students in their investigations of the relationship between structural and biochemical correlates of developing embryos.

Over the last decade, the use of inquiry-based collaborative learning in the classroom has engendered a new approach in pre-college (National Research Council, 2000) and college laboratories. The National Science Foundation (NSF) emphasized that a rapid accumulation of scientific knowledge, changes in workforce requirements, and lack of scientific literacy in the general population are sufficient reason to revisit our science curricula. NSF proposes inquiry-based experiences, in which students perform steps that scientists employ as they answer research questions. The students come to "own" the research project (NSF, 1996). According to Drayton and Falk (2001), inquiry-based learning is characterized by various elements: learners are engaged by scientifically oriented questions; they give priority to data in order to address and develop tenable hypotheses and use cogent arguments to convince others of their explanations. One of us has used a similar approach in a physiology course with promising results (Hoegler, 2005). Here, the weekly schedule of laboratories was divided into 2 parts: one, prescribed (Phase I) earlier on in the semester and the second, more student-generated (Phase II) during the last month. During Phase I, after students learned the processes for performing several experimental techniques; they applied the skill sets in various prescribed exercises in small groups of 3 or 4, under the professor's guidance. They gathered data from these experiments and presented an analysis of the data before the class using PowerPoint. They also learned the elements of library searching of databases to refine their skills in exploring new avenues of research. During the last month of the course, student teams progressed into Phase II. Therein, they researched the databases to find available literature about a topic of interest and designed a project tailored to their interests. A learning pyramid shows how skills from the first part of the course provide a foundation for developing a research proposal (Fig.1). This approach should encourage curiosity. It will also challenge the critical thinking skills of the students. These proposals will be examined critically and expeditiously; if

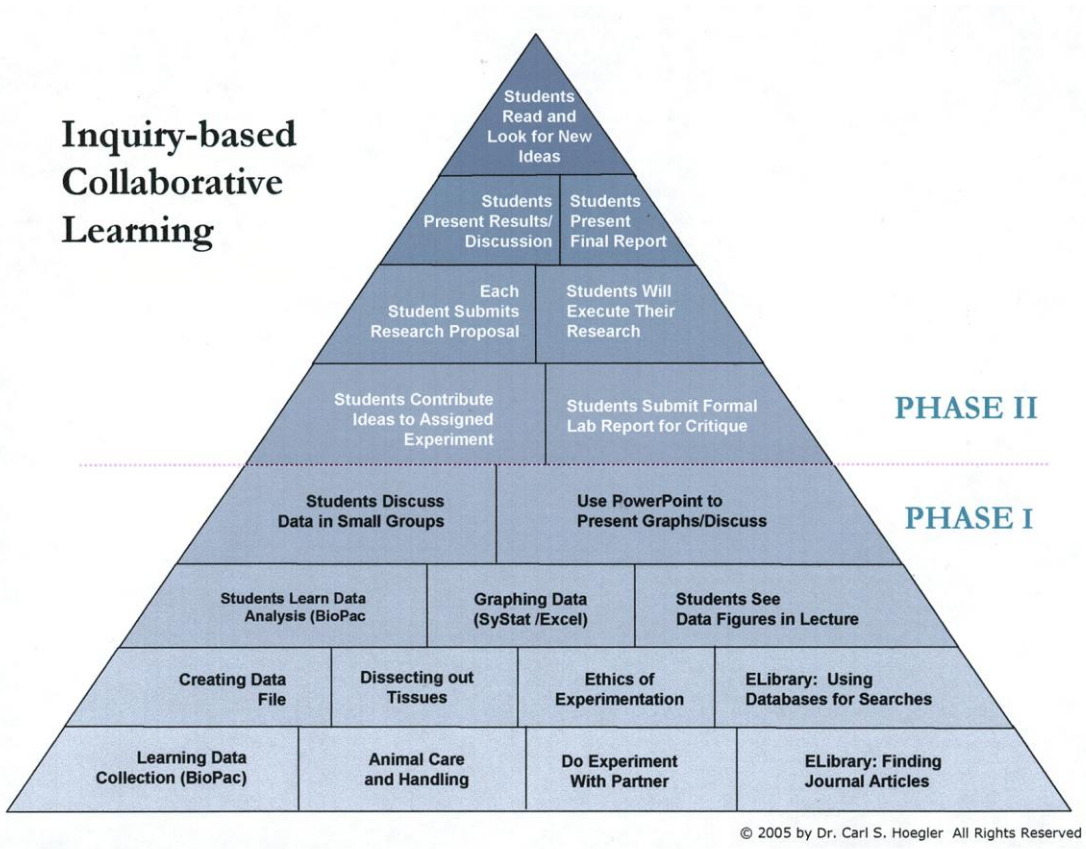

Figure 1. Learning pyramid showing student activities during Phase I and Phase II of inquiry-based collaborative learning (Hoegler, 2005). Earlier in the semester students first perform activities at the base of the pyramid before reaching higher levels

needed some suggestions could be provided for revisions. Student teams will then carry out their proposed experiments in the lab, collect data and present their report to the professor and classmates.

In this paper, we are suggesting one possible exercise that could be integrated into the laboratory in a Developmental Biology course. In our proposal we have used normally developing ranid frogs, comparing them to spontaneously abnormal specimens. Both *Xenopus* and *Rana* are commercially available and reasonably priced and will provide a source of embryos used in the student experiments. Basically we are proposing that student teams will be encouraged to investigate the effects of some physical or chemical agent on amphibian embryogenesis. Students will read articles about effects of environmental chemical agents on amphibian development. After database search, they will consider information about environmental disturbances and then integrate these into their proposal. In the past, our students have suggested learning about the effects of acid ("acid rain") and gasoline additives because of popular interest by the media. In this proposal students will pursue more than morbidity and mortality rates. They will look at the effects on the embryonic protein banding patterns. Digital photography would be used to document any phenotypic morphological effects. Students will also analyze the protein banding patterns to determine any correlative changes in the biochemistry. In summary, we are suggesting a more exciting and challenging open-ended approach to developing lab exercises for undergraduate education.

Materials and Methods

A. Rana pipiens as a model organism

Frog embryos are available and reasonably priced so students can test a variety of hypotheses. For the purposes of this research, students will use *Rana pipiens* to obtain embryos by combining eggs, squeezed from a gravid female with sperm from macerated testes. Detailed instructions are provided by Roberts Rugh (1962) in his classic text. Students will have some choice of directing their projects by altering developmental parameters such as: pH, temperature, chemical inclusions in the growth environment, radiation etc. Embryonic effects can be documented using photography, total protein electrophoresis and Western blotting for identification of specific proteins.

At specific early stages of development (Shumway, 1940) embryos will be harvested; stages before hatching will have their jelly coats removed. After collection, embryos will be frozen and later pulverized in distilled water.

B. Protein Calibration of embryo homogenates

The total protein of each sample was determined to equilibrate protein levels across wells of the PAGE gel. *Bio-Rad* protein assay required the use of visible light spectroscopy (595nm). A calibration curve is first developed using a known protein, such as bovine serum albumin (*Sigma-Aldrich*). The optical densities (OD) of the embryonic extracts were compared along the standard curve to determine quantity of protein. Use of 1ml cuvettes minimized the quantity of extract. Reliable optical density (OD) readings are usually found along the lower range of concentrations of BSA. Eventually the embryonic extracts are treated with mercaptoethanol (ME) in Laemmeli buffer and prepared for SDS-PAGE (see C. below).

C.PAGE (Polyacrylamide Gel Electrophoresis)

The technique of electrophoresis is generally known to most biologists and the protocols may differ slightly from one lab to another. A published protocol can be found in a paper from a previous ABLE Conference (Racusen and Thompson, 1996).

The electrophoresis apparatus, power source and transphor unit were purchased from *Bio-Rad Laboratories, Inc*. Just before electrophoresis, samples in microfuge tubes were thawed in tap water and re-pulverized over ice with a thin glass pestle (engineered from a Pasteur pipette). The samples were then vortexed and centrifuged for 10 minutes in a *Serofuge* centrifuge and supernatant was decanted. Aliquots of the tissue extract were diluted 1:1 with Bio-Rad sample buffer (mixture of 950µl Laemmli buffer and 50µl of mercaptoethanol). The mixture was heated at 95° C for 5 minutes and then placed in an ice bath.

Precast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (15%) were inserted in a *Bio-Rad* gel cassette sandwich of the Protean 3 electrophoresis system, which slides into the electrode assembly. Detailed instructions can be obtained from the company. Pre-mixed Tris-Glycine buffer (*Bio-Rad*) is diluted and poured into upper and lower chambers. Then the wells are loaded. The first lane has a protein ladder with *Bio-Rad* Protein Plus markers (6.5- 205 kD). It is recommended that about 20-25µg protein equivalent be loaded across wells. Thus, the delivered volumes per well may vary according to the protein concentration in the sample (see B. above). You

should also readjust this concentration after diluting with the sample buffer. Remember that in purchasing the precast gels, you should designate the appropriate volume capacity of the wells.

Once loaded, the gels are run at 180 V for approximately 45 minutes. Check that the tracking dye does not exceed the gel length! After the run, the gel is removed and notched at one end. After several washes in distilled water, it is stained in commercially available *Bio-Rad* Coomassie blue solution for 1 hour (this stain can be re-used). Destaining with distilled water over the next 2-3 days is absolutely essential for clarifying the bands.

D. Western Blotting

Gels that were to be Western blotted were not immediately stained. They were placed in blotting buffer and then assembled into a sandwich with nitrocellulose membrane, filter paper and fiber pads. This is slid into the gel holder cassette of a *Mini Trans-Blot* apparatus (*Bio Rad)*. The directions for preparation of the blot are provided in elegant detail in an instruction manual provided by the *Bio-Rad* Laboratories (2005). During the 2.5 hour run at 20V, blocking buffer, primary (anti-MLC monoclonal), secondary (goat-anti-mouse polyclonal (conjugated with horseradish perioxidase-HRP) antibody and chromogen (HRP color detection kit) are prepared. After the run, overnight blocking is suggested but our research reveals that 15-20 minutes of blocking and rapid washes ensured that less of the transferred protein would be lost. It is very important to have good rocking action on the platform rocker for proper blocking, rinsing and color development of blots. The use of the primary and secondary antibodies is followed by adding the HRP substrate (in the dark) and allowing the color to develop to reveal the presence of the targeted protein.

E. Data Analysis

A *NucleoVision* imaging system was used on gels and blots. After scanning and pre-processing the image, it was possible to determine the position and intensity of the bands. SONY printing equipment and software displayed the stained gel for analyses. The scanning of the nitrocellulose blots can be done in similar fashion.

Since commercially available imaging systems are quite expensive, an alternative approach to analysis would involve digital photography of the gels after a run; images are saved as .jpg or .tif files. Photo-editing software clarifies the protein bands. After the photos are annotated to describe the lanes, migration lengths can be measured. Direct comparisons of bands can be standardized by calculating Rf values relative to the origin. The use of a molecular weight ladder of known protein standards allows students to estimate band molecular weights. Similarity index can also be used to compare differences in banding patterns between samples (Vaughan and Denford, 1968).

Results

Our results suggest that there are clear differences between the morphology of normal and viable but spontaneously abnormal embryos. The most prominent difference is a bent tail syndrome, in which the tail fin is rigidly flexed ventrally; this becomes evident at the later collection stages including tail fin (TF), gill circulation (GC) and opercular fold (OP) (Fig. 2).

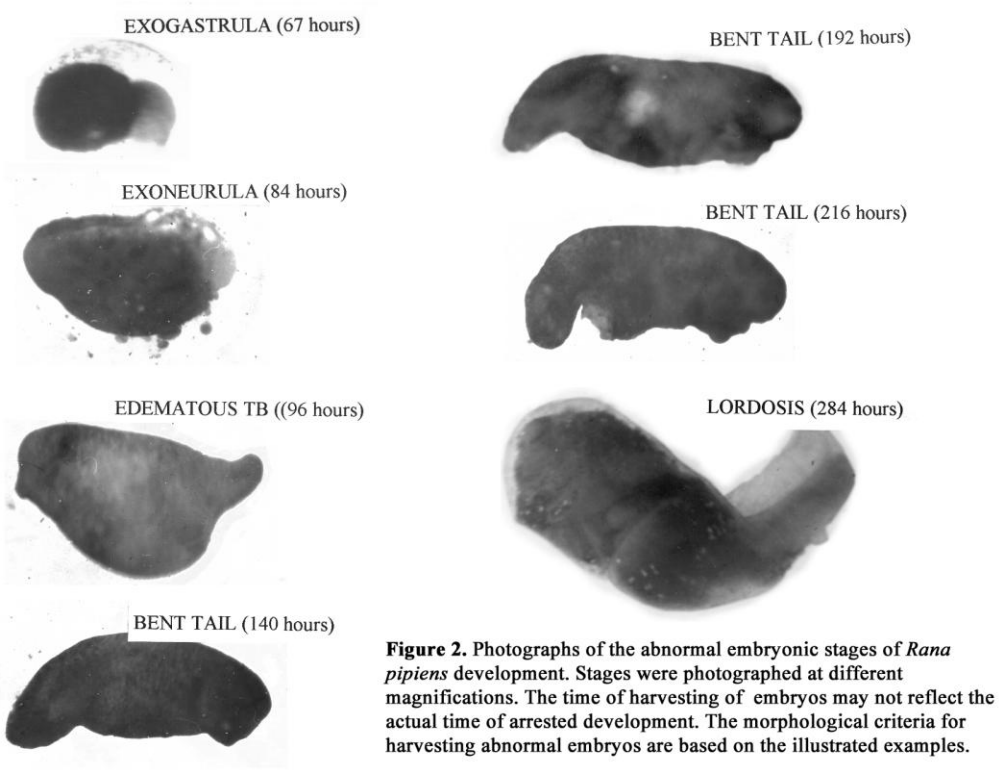

Figure 2. Photographs of the abnormal embryonic stages of *Rana pipiens* development. Stages were photographed at different magnifications. The time of harvesting of embryos may not reflect the actual time of arrested development. The morphological criteria for harvesting abnormal embryos are based on the illustrated examples.

Extracts of normal embryos reveal subtle differences in the protein band pattern as embryogenesis progresses. There was an overall pattern of similarity over time but subtle alterations were visible (Fig.3). Spontaneously abnormal *Rana pipiens* embryos, grown in spring water where no evident toxins are present, appear to show differences in protein banding, compared with normal embryos. Thus, when compared to normal stages, the total protein banding from these abnormal embryos at GC, TF and OP were distinctly different. Protein gel analysis of the banding intensities of normal developing embryos revealed more protein bands, with greater intensities (Figs.4 TOP, 5 TOP) than in abnormal embryos. This was especially evident at GC, TF and OP stages of embryogenesis.

These results were demonstrated across 4 different electrophoretic runs using 2 different sample preparations. The embryo extract preparations were easily performed and the samples appeared to sustain their protein banding patterns months after initial collection provided that the tissues are stored under frozen temperatures. This feature might be useful when preparing samples for multiple-section groups of students.

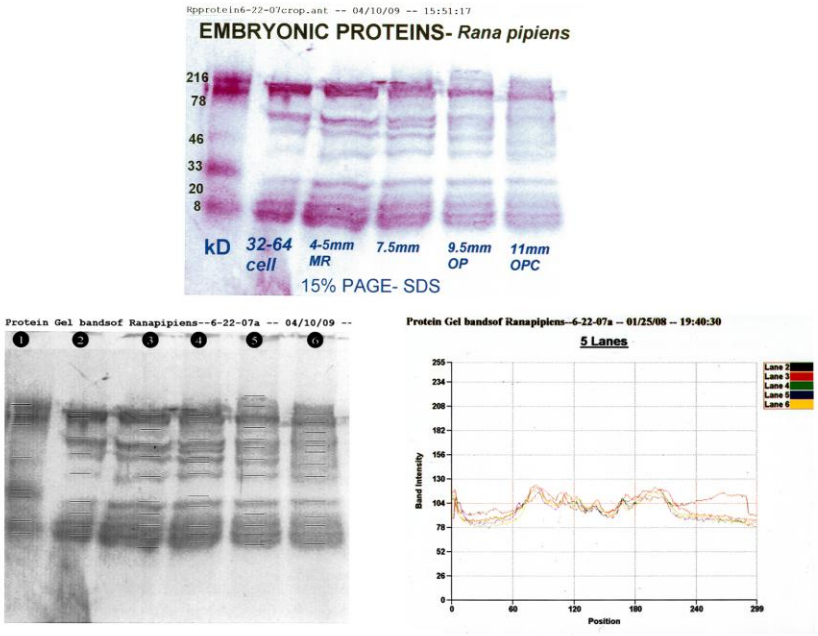

Figure 3. Protein banding (SDS-15%PAGE) of normal embryos harvested at 5 different embryonic stages (*Rana pipiens*) (cleavage=32-64 cell stage; MR-muscular response-4-5mm tadpole stage;7.5mm tadpole;OP-opercular fold-9.5mm tadpole stage and OPC-opercular complete-11mm tadpole stage) (TOP). Corresponding photographs of embryo shown in fig 4. Shumway(1940).Note the similarity of the banding patterns among the scanned lanes (BOTTOM)

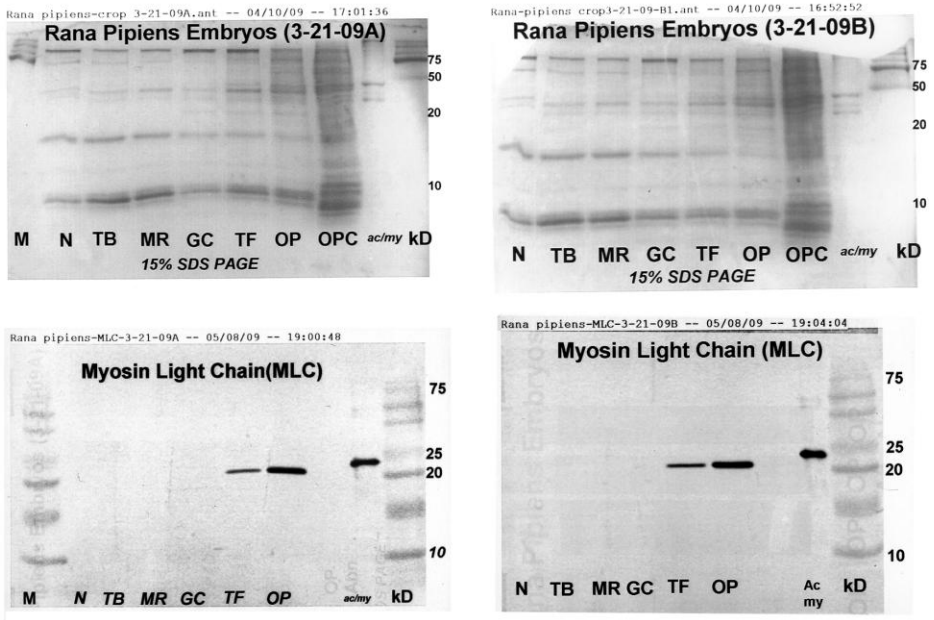

Figure 4. Protein banding (SDS-15% PAGE) of normal Rana embryos(TOP) harvested at 7 different stages of embryonic development (N=neurula; TB=Tailbud; MR=Muscular Response; GC=Gill circulation, TF=Tailfin; OP=Opercular Fold; OPC=Opercular Complete) . BOTTOM shows evidence of Myosin light Chain(MLC) at 2 stages(TF, OP).

There was also evidence of myosin light chain (MLC) at tailfin (TF) stage, and again at opercular fold (OP) in normal frog embryos (Fig.4, BOTTOM). However, the presence of MLC as a single dark band was detected only at OP stage in abnormal developing embryos (Fig.5, BOTTOM), and not at tailfin.

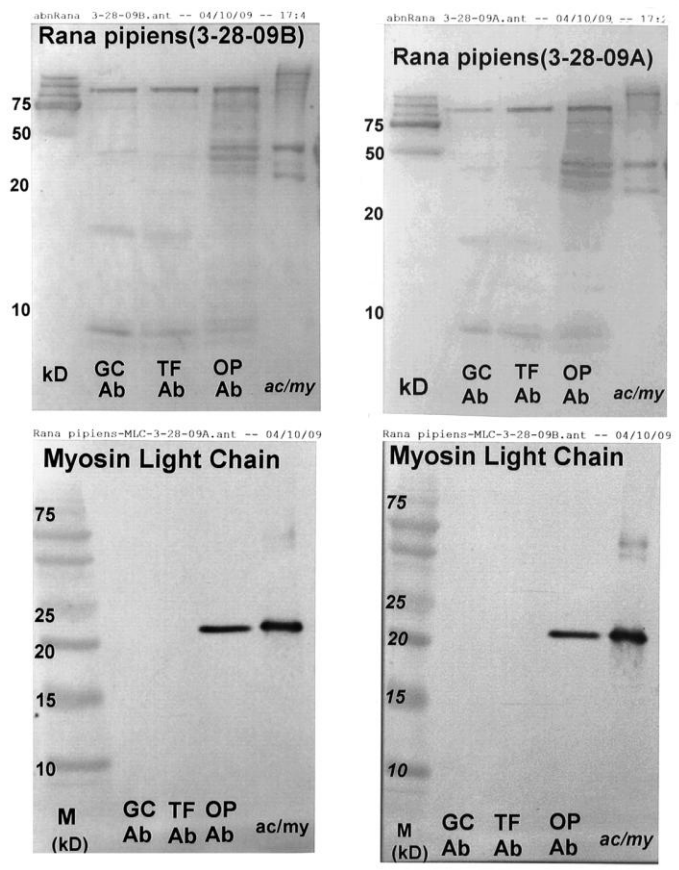

Figure 5. Protein banding (SDS-15% PAGE) of abnormal Rana embryos(TOP) harvested at 3 different times of embryonic development (GC=Gill circulation, TF=Tailfin; OP=Opercular Fold). Harvesting time does not necessarily correspond to the stage of the arrested/abnormal development, since their onset might have preceded the time of harvest. BOTTOM shows evidence of Myosin light Chain (MLC) appearing at one late stage (OP).

Discussion

In conclusion, it appears that students could enrich their understanding of developing systems by looking at both morphological and biochemical aspects of embryogenesis. Using IBCM (inquiry-based collaboration model), instructors would prepare students during Phase I with library exploration and research methodologies in molecular biology. The latter skills would include culturing, harvesting and staging amphibian embryos as well as extract preparation, SDS-PAGE electrophoresis, Western blotting and data analysis. Embryo culturing and embryo extract preparation are rather routine and not likely to trigger student protest. This approach gives the students practice in making extracts, protein equilibration, electrophoresis and analyses of banding patterns from animal tissues. And finally, studying the protein biochemistry of developing systems may lead to further investigations of the regulation of gene expression.

In Phase II, students, working as teams, would design a research proposal that utilizes their learned skills to investigate some aspect of developmental biology, perhaps using another amphibian

model or other teratogens on the *Rana pipiens* model. Such exploration could introduce excitement to a course, since it gives the student more autonomy in tailoring her/his learning approach to the interests of the group. IBCM will also help the instructor to assess the ability of individuals in a team, to integrate skills and knowledge. This approach may serve as a capstone experience for some schools. Alternatively, the research project of each team could be displayed at a departmental poster session.

There are caveats in the methodology. For example, harvesting time of abnormal embryos does not necessarily correspond to the stage of the abnormal embryos because the onset of abnormal development might have preceded time of harvest. Also, chronological age of the abnormal embryo is difficult to track. Statistics on the proportion of abnormal could have also been performed.

Nevertheless, the etiology of these abnormal but spontaneous changes should promote interesting class discussion. For example, are the spontaneous abnormalities in our study due to minute quantities of chemical agents that are otherwise undetectable? To what extent does crowding influence normal development? Do normal tadpoles release substances that affect growth of other tadpoles? Are abnormalities due to genetic causes (including epigenetic expression)?

The obvious coincidence or divergence of some banding patterns among various developmental stages of *Rana pipiens* embryos could have been corroborated by quantitative *similarity index (S.I.)* (Vaughan and Denford, 1968). The phenotypic changes seen in embryonic photographs are tracking key morphological events (N=neurula; TB=tailbud, MR=muscular response; GC=gill circulation; OP=operculum forming; OPC=operculum complete) which may or may not reflect changes in protein banding profiles. This seems noteworthy because the gel profiles of abnormal embryo development resemble, in many aspects, protein profiles of normal embryo development. This necessarily requires an understanding of the kinetics and biochemistry of embryogenesis.

The inquiry-based collaborative model (IBCM) should enable students to take greater responsibility for understanding. However, this novel approach requires superior commitment from the instructor. They must be willing to work many hours with the student teams who will challenge them with a variety of different projects. Some understanding must be reached about budgetary allowances for the different groups. Students will require several weeks for the research to be accomplished, and for the PowerPoint or poster to be designed and presented. The long-term promise of this approach could be clearly realized in students who are considering graduate education as suggested by another study (Hathaway et al. 2002). At that level of higher education, students are often given less direction and they are expected to jump start projects by tapping their intellectual curiosity and creativity. An enriched undergraduate experience such as in IBCM might provide the confidence to tackle this future challenge.

It is also suggested that, because of the extra lab time, the instructor might wish to re-evaluate whether she/he should introduce the Western blotting to the entire class. This step also requires significant expense, due primarily to the cost of antibodies. Nonetheless, it is our hope to use this proposed study to incorporate IBCM in a future Developmental Biology course.

Acknowledgements

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