An Inexpensive Method to Simulate a Monohybrid Cross Using Wild-Type Zebrafish (*Danio rerio*)

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Abstract: Monohybrid crosses are taught in biology labs across the country using peas and corn. Students studying monohybrid crosses using zebrafish (*Danio rerio*) would encounter a new model organism, and those interested in human or veterinary medicine would have increased interest. To this end, our college has implemented a laboratory exercise at the introductory level using wild-type and non-pigmented zebrafish embryos to simulate a monohybrid cross. By using 1-phenyl-2-thiourea (PTU) to generate non-pigmented fish, the necessity of maintaining a separate albino line of fish is eliminated. Live embryos are preferable for generating student excitement; however, the option to use preserved embryos allows for re-use of fish over multiple years without relying on embryo production on a particular day.

***Keywords*:** Mendel, zebrafish, monohybrid, genetics

Introduction

In the classroom, we are often challenged with presenting material in new and exciting ways. Monohybrid crosses in particular can be difficult to present in a novel way in an introductory college biology course for either majors or non-majors. Mendel famously used the pea, *Pisum sativum*, to discover the 3:1 phenotypic ratio of dominant:recessive traits (Mendel, 1866). Many high schools and colleges continue to use this model organism, as well as corn, to exemplify genetic crosses. In our introductory biology course serving a population of approximately 120 students per year, we have used corn to demonstrate both monohybrid and dihybrid crosses (Genetics of Corn Kit, Carolina Biological).

Students often have preconceived notions brought into the classroom and laboratory environment (Lazarowitz and Lieb, 2006). While not the intent of educators, some students may receive the impression that monohybrid genetic crosses are the domain of plants after exploring the concept exclusively in corn. Those students with prior exposure to Punnett squares and Mendelian genetics would benefit from new examples. In addition, critical thinking skills can be developed in laboratory exercises through their inquiry-based nature (Howard and Miskowski, 2005). Here I will describe a laboratory exercise involving a simulated monohybrid cross in zebrafish (*Danio rerio*) to complement common Mendelian laboratory exercises involving corn.

This laboratory exercise emphasizes that the phenomenon of 3:1 phenotypic ratios can be found in animals as well as plants. In addition, the exercise introduces a common model organism to students. Finally, the exercise simulates albino zebrafish without the instructor having to maintain a line of albino fish. Therefore, the exercise is adapted for high schools and colleges having minimal zebrafish facilities and circumvents the need to maintain multiple genetic lines. While live embryos are the most advantageous for student interest, preserving the embryos ahead of the lab exercise allows for specimens to be used multiple years and without relying on embryo production on a particular day.

Materials and Methods

Wild-type zebrafish adults (*Danio rerio*) were purchased from Carolina Biological and housed in ten gallon tanks. Adults were kept on a 14h:10h day:night cycle at 28.5°C and fed a diet of Tetramin flakes and freeze-dried brine shrimp (Omega One). Approximately 20 fish were bred over a collecting tray and embryos were collected 2 hours after simulated dawn. Embryos were transferred to 60 mm Petri dishes with 0.3x Danieau solution (Table 1) and kept at 28.5°C (Nasevicius and Ekker, 2000).

At 7 hours post fertilization (hpf), clutches of embryos were placed into either a new Petri dish of 0.3x Danieau or 0.3x Danieau containing 0.2 mM 1-phenyl-2-thiourea (PTU). PTU blocks melanin synthesis resulting in embryos that appear albino (Karlsson *et al.*, 2001). Over the course of 3 weeks, approximately 75% of embryos were placed into 0.3x Danieau and 25% of embryos were placed into the solution containing PTU (Figure 1). Mating of adult zebrafish was continued until approximately 200 embryos were obtained for each student group in a lab section. For our class of 120 students, no more than four lab groups were in a session at any one time, so 800 embryos were needed.

Table 1. Recipes used in zebrafish Mendelian genetics laboratory exercise

Reagent	Recipe
50x Danieau buffer stock	2.9 M NaCl 35 mM KCl 20 mM $MgSO_4$ 30 mM $Ca(NO_3)_2$ 250 mM HEPES pH 7.6
100x 1-phenyl-2-thiourea (PTU) stock	120 mg PTU in 40 mls 0.3x Danieau buffer (20 mM) Freeze in 5 ml aliquots
0.3x Danieau buffer	3 mls 50x Danieau buffer 497 mls H_2O
0.3x Danieau buffer with 1x PTU	3 mls 50x Danieau buffer 5 mls 100x PTU stock 492 mls H_2O
4% Paraformaldehyde (PFA)	0.4 g PFA 1 ml 10x Phosphate Buffered Saline (PBS) 9 mls H_2O
PBT wash	5 mls 10x PBS 50 ul Tween 20 45 mls H_2O

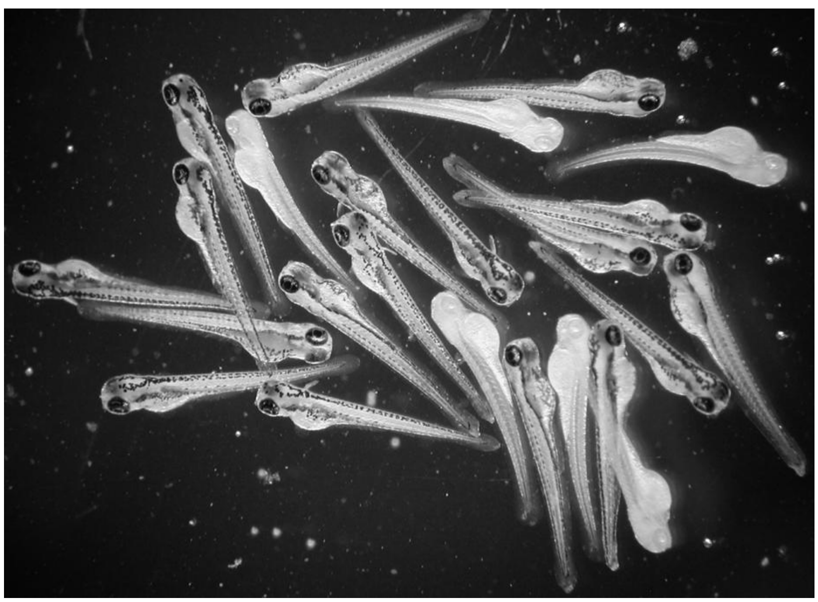

Figure 1. Clutch of 72 hpf embryos with approximately 25% treated with PTU. A ratio of approximately 3:1 wild-type:non-pigmented embryos is used to simulate a monohybrid cross between wild-type and albino zebrafish.

At 24 hpf, dead embryos were removed from Petri dishes to maintain a clean environment for surviving specimens. This removal of dead embryos is common in the zebrafish field. Mortality in zebrafish embryos is variable depending upon egg and sperm quality. PTU did not affect mortality. At 72 hpf, embryos were transferred to a glass vial and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C. Embryos were then washed three times for 5 minutes each in PBT at room temperature (Table 1). Embryos were sorted into an approximate 3:1 phenotypic ratio of wild-type:non-pigmented and stored at 4°C in glass vials. To accurately simulate a natural mating, the ratio was not exactly 3:1. Immediately before the laboratory procedure began, embryos were transferred to covered 60 mm Petri dishes containing PBT

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Laboratory Procedure

The first exercise in the laboratory (not shown here) introduced the terminology of dominant and recessive alleles using the Genetics of Corn kit from Carolina Biological mentioned above. In brief, students examined purple and yellow corn kernels and counted to find an approximately 3:1 phenotypic ratio of purple:yellow corn kernels. Students were asked if their observed values were near expected values, and introduced to the Chi-Square test for Goodness-of-Fit. Students then proceeded to the zebrafish exercise described below.

The zebrafish exercise began with students observing a wild-type adult fish. Then, using a stereomicroscope, students were asked to observe a preserved wild-type zebrafish embryo. Both of these observations allowed us to introduce students to the zebrafish model organism, use of a stereomicroscope, and basic embryo anatomy (head, eye, tail, yolk sac). After this familiarization was complete, students used the stereomicroscope to count approximately 200 embryos (in one Petri dish) for wild-type and non-pigmented phenotypes. Results were then analyzed by the Chi-Square test for Goodness-of-Fit. The laboratory exercise lasts 30-40 minutes. Students then proceeded to dihybrid crosses using the Genetics of Corn kit mentioned above.

For the purposes of the assignment, non-pigmented embryos were called albino. Instructors may or may not choose to communicate the melanin inhibition to students. If the instructor does not, he or she should make sure to indicate that these fish are phenotypically albino but not genetically albino. A Chi-Square table was provided with the exercise.

Student Assignment

1. You have tested one of Mendel's Principles on a plant. Does his Principle of Segregation also apply to animals? You will test this question using pigmented and albino zebrafish. Obtain a live zebrafish adult, representing the F_1 generation of a cross between a true-breeding pigmented fish and a true-breeding albino fish. Which phenotype is dominant?

2. Draw a Punnett square showing the cross of a true-breeding pigmented fish (AA) with a true-breeding albino fish (aa). What genotype does the F_1 fish have?

3. Draw a Punnett square showing a cross between two F_1 fish. What are the expected genotypes (and ratios)? What are the expected phenotypes (and ratios)?

4. Obtain 3-day old preserved zebrafish embryos from your instructor. The Petri dish contains a clutch of embryos, all siblings. Observe the embryos. Can you see the eye? The heart? Record the embryo pigment phenotype counts in Table 2 under the "observed" column.

5. Calculate the expected number for each phenotype based on the ratio in part 3. For example if you expected a ratio of 8 pigmented:1 albino and counted 180 embryos, you would expect (8/9)*180 embryos to be pigmented (160 embryos) and (1/9)*180 embryos to be albino (20 embryos). Record the expected numbers in Table 2 and complete the Chi-Square calculation.

Table 2. Chi-Square calculation of F_2 zebrafish pigmentation phenotypes

	observed	**expected**	**observed-expected**	**(observed-expected)^2 /expected**	**(observed-expected)^2 /expected calculated**
Pigmented					
Albino					
Sum					

6. State your null hypothesis before checking your p-value.

7. Check the Chi-Square table for your p-value and interpret your results. Write a well developed paragraph that addresses the following: What are you testing (what was your expected phenotypic ratio and why)? What is the observed phenotype ratio? Is it near your expected? Do you accept or reject your hypothesis? Why or why not? Explain your reasoning.

Laboratory Evaluation

Approximately 85% of students either thought the laboratory exercise was fine or liked it a great deal (Figure 2). Students felt that both zebrafish and corn were equally effective in four areas assayed (Figure 3), but enjoyed the zebrafish portion of the exercise more than the corn portion (Figure 4). Only 11% of students had used a vertebrate in a high school genetics lab exercise. A free-form question asked what students liked best and least about the complete laboratory exercise, which included corn and zebrafish exercises. The highest percentage "liked best" answer was zebrafish (40.5%), while 14.4% liked corn the best. In contrast, 7.6% liked zebrafish least, while 8.7% liked corn least. The highest percentage of any "liked least" free-form answer was the Chi Square test at 29.3%.

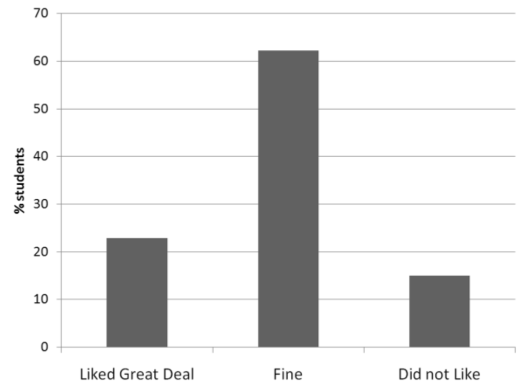

Figure 2. Assessment of student enjoyment of the complete laboratory exercise, including both zebrafish and corn portions. Most students liked the lab a great deal or thought the lab exercise were fine.

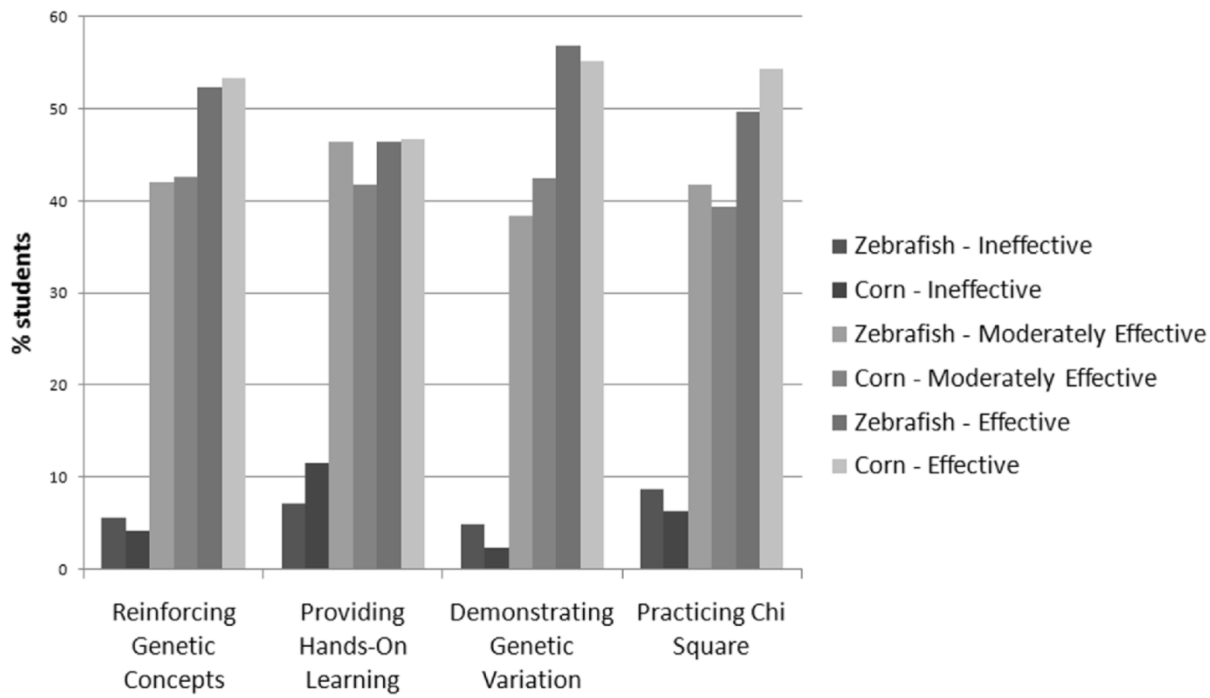

Figure 3. Assessment of laboratory exercise effectiveness, as reported by students. Students rated the corn and zebrafish laboratory exercises as equally effective.

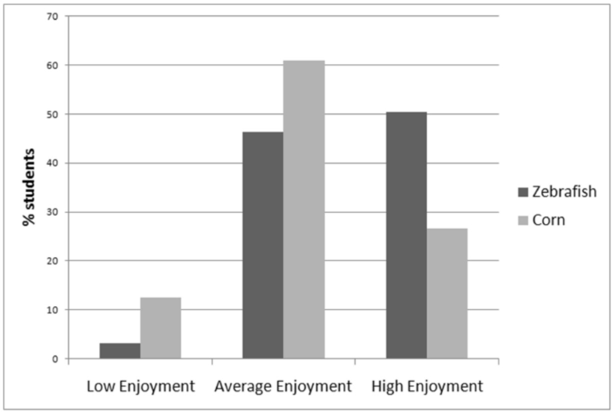

Figure 4. Assessment of student enjoyment of the zebrafish and corn portions of the laboratory exercise. Students rated the zebrafish exercise as "high enjoyment" nearly twice as much as the corn exercise.

Discussion

For many students, Mendelian genetic principles, when combined with Chi-Square calculations, are daunting. While the often-used corn laboratory exercise provides an invaluable method for introducing monohybrid and dihybrid crosses, our college wants to stimulate student interest in genetics by using an animal model. Many of our prospective undergraduate biology majors are interested in careers in human or veterinary medicine. Introducing zebrafish as a model system at this level engages those students. In addition, this encounter with embryos is often the first for many students. Reigniting the spark of curiosity in students is a primary concern for educators.

Students participating in the laboratory exercise were excited about using an animal model. We found that 89% of our students had not worked with a vertebrate animal model in a genetics laboratory setting during high school. In the qualitative portion of the survey, one student enjoyed learning about Mendelian concepts in animals rather than plants. Another indicated that zebrafish were the primary reason that the lab held his or her attention. Students did not rank the zebrafish as more effective than the corn examples provided during the laboratory exercise, but expressed "high enjoyment" with the zebrafish more than twice as much as the corn portion of the exercise. We provided live adult zebrafish, which the students found useful to visualize the adult form of the embryos used.

Students did encounter two problems with the laboratory exercise. Some students were hesitant when asked to identify embryo anatomy (such as the heart, yolk sac, or eye) before looking under the microscope. Zebrafish embryos have readily identifiable organs, such as the eye and heart, which students were able to identify once they engage with the provided materials. The use of zebrafish embryos in introductory biology courses has become more widespread (D'Costa and Shepherd, 2009). Another common problem was unfamiliarity with the Chi Square Goodness of Fit test, the least liked portion on the qualitative portion of the survey. Students did perform the test once before (a monohybrid corn cross used immediately before the zebrafish exercise), and the exercise discussed here increases familiarity with the test by repetition. Students go on to perform the test on sex-linked *Drosophila* crosses to find other genotypic ratios and to discover parental genotypes the following week. By repetition and instruction, most students are able to grasp the Chi Square test by the end of the two weeks of genetic laboratory exercises.

Active learning exercises such as laboratories are important in introductory biology courses (Smith *et al.*, 2005). Laboratory exercises are almost always active learning experiences and are important in building critical thinking skills. These skills are desired in Bloom's taxonomy of critical thinking and can help students perform better on exams (Chaplin, 2007). In addition, the exercise described here integrates mathematics and statistics into the biology classroom, which is often desirable (White and Carpenter, 2008). Asking questions and using statistical tests begins the important process of incorporating inquiry and research into the curriculum.

In conclusion, our college has implemented a zebrafish laboratory exercise to complement a well-established corn genetics laboratory. This laboratory exercise simulates a monohybrid cross between wild-type and albino zebrafish using PTU to inhibit melanin synthesis. Because the cross is simulated, this exercise can be done for low cost (only the cost of PTU) compared with maintaining an albino line of zebrafish. Students enjoyed the hands-on aspect of working with a vertebrate in a genetics laboratory. Finally, the preserved embryos can be used over multiple laboratory sections and for many years.

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