A class discussion of the results of these crosses could include the following topics: 1) Why was the inter-isofemale average viability of generation nine (206 progeny) below the average viability of the generation one flies (211)? The 19 lines used in this study all came from one location and may have shared some deleterious alleles. Hence, in some inter-isofemale crosses a few deleterious mutations may have still been homozygous. 2) If crosses had been made for 19 lines collected from 19 separate locations, how might this have changed the generation one and inter-isofemale results? In this case, one would expect few, if any shared deleterious alleles in the lines. 3) Discuss how organisms in nature, including mammals, avoid inbreeding depression. How do humans avoid inbreeding depression?

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Genotyping and enzyme activity measurements of the *Adh* polymorphism: a simple exercise in population genetics, biochemistry, and the connection of genotype and phenotype.

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Introduction

The study of genetic variation at enzyme loci offers students insight into population genetics, protein molecular evolution and structure-function relationships, and the connection between genotype and phenotype. An excellent teaching example is the *Alcohol dehydrogenase* (*Adh*) amino acid polymorphism in *Drosophila melanogaster*. This polymorphism is one of the most studied in modern molecular genetics; a textbook example of genetic variation likely under selection, it has been the focus of a number of seminal works in the study of molecular evolution (*e.g.*, Oakeshott *et al.*, 1982; McDonald and Kreitman, 1991). There are two *D. melanogaster Adh* alleles that can be resolved by protein electrophoresis. The fast (ADH-F) and slow (ADH-S) allozymes, named so for their electrophoretic migration rates in standard starch-gel electrophoresis, differ by a single threonine to lysine substitution (Fletcher *et al.*, 1978; Kreitman, 1983). The alleles also differ in V_{max}: ADH-F alleles generally have higher activity than ADH-S alleles, but possibly at the expense of decreased stability in higher temperatures (Fletcher *et al.*, 1978). Reciprocal latitudinal clines and non-random patterns of inter- and intra-specific genetic variation suggest the alleles are under selection (Oakeshott *et al.*, 1982; McDonald and Kreitman, 1991).

ADH functions as a dimer facilitating biological detoxification through the conversion of alcohols to ketones and aldehydes (*e.g.*, ethanol to acetylaldehyde, using NAD^+ as a reducing cofactor). Flies homozygous for the *fast* allele (*Adh-F/Adh-F*) show a better ability to survive on ethanol as an energy source, possibly as a result of higher ADH activity (Daly and Clarke, 1982). Interestingly, both alleles are naturally maintained in wild populations with allelic frequencies shifting along latitudinal clines such that the *Adh-F* allele increases in frequency at higher latitudes (Oakeshott *et al*., 1982; Berry and Kreitman, 1993), suggesting that each allozyme may have a certain advantage over the other in different environments.

In this teaching protocol, students genotype individual flies for the ADH polymorphism (*Adh-F/Adh-F, Adh-F/Adh-S, Adh-S/Adh-S*), test the observed genotype frequencies for neutrality based on expectations from Hardy-Weinberg equilibrium, and examine connection between genotype and phenotype by characterizing the ADH enzyme activity of each fly. The protocol described here is an expansion of Thompson *et al*.'s protocols (2000, 2006), from which it draws heavily. Students examine the *Adh-F* and *Adh-S* alleles within a wild population of *D. melanogaster*, easily and inexpensively genotyping each fly using cellulose-acetate gel electrophoresis. ADH enzyme activity in each fly, the oxidation of an alcohol (ethanol or isopropanol), is quantified by measuring the increase in absorbance at 340nm as the metabolic cofactor NAD^+ is converted to NADH. The relationship between genotype and enzyme activity can then be examined using simple graphing and statistical methods. The cellulose acetate electrophoretic separation used in this protocol results in a reversal in migration rates of the ADH alleles compared with the traditional, starch gel-based, electrophoresis. The traditional "slow" allele runs farther than the traditional "fast" allele due to a difference in buffer composition. The results, therefore, must be "translated" back to traditional ADH-F and ADH-S alleles for comparison with the literature.

Materials and Methods

Fly preparation

The flies used in this exercise are collected from isofemale lines of *D. melanogaster* established from a local population. As an added dimension to the lab, students can trap local flies and can either establish and maintain their own fly lines or directly assay individual flies. Using CO_2 to anesthetize the flies, individual flies are chosen at random and are placed in 1.5 mL microtubes for homogenization. Microtubes are kept on ice to keep the flies anesthetized and prevent protein degradation after homogenization. Each fly is homogenized using a disposable tissue homogenizer for only a few seconds in 50 µL of Tris-Glycine running buffer, pH 8.5 (TG Buffer: 30g Trizma-base [Sigma-Aldrich, cat. #T1503] and 144g Glycine [Sigma-Aldrich, cat. #G7126], made up to 1L for a stock solution; to use, dilute 1:9 TG:water [Hebert and Beaton, 1993]). Microtubes containing the fly homogenate are centrifuged (5000G, 1 min., 4°C) to pellet all solids and the supernatant is transferred to new tubes.

Genotyping

The *Adh* genotype of each fly is determined by electrophoretic separation and enzyme-specific staining of the individual whole-fly homogenates. 10 µL of homogenate is transferred onto a well on the Sample Loading Plate (Helena Laboratories, cat. #4085) and, from there, loaded onto the cellulose acetate sheets (Helena Laboratories, cat. #3023) for electrophoretic separation of the alleles. Cellulose acetate sheets are presoaked in TG running buffer for at least 20 minutes prior to electrophoresis. Individual cellulose acetate sheets are transferred to the aligning base (Helena Laboratories, cat. #4086) with the mylar side down and blotted to remove excess buffer. The homogenate from the Sample Loading Plate is transferred onto the cellulose acetate sheet using an

applicator (Helena Laboratories, cat. #4084). One cellulose acetate sheet is capable of running two rows of 8 samples.

The cellulose acetate sheet loaded with fly homogenate is transferred onto conductive wicks (Helena Laboratories, cat. # 5081) in an electrophoretic gel tank (Helena Laboratories, cat. #4063) with the mylar side up and with TG running buffer in both the anode and cathode portions of the tank. Electrophoresis is carried out at 200 volts for 20 minutes. Cellulose acetate sheets are stained with an ADH staining solution (600 µL of 0.09M Tris-HCl, pH 7.0 [Sigma-Aldrich, cat. #T1503], 1.5 mL of 4.0 mM NAD^+ [Sigma-Aldrich, cat. #N7381], 150 µL isopropanol [Sigma-Aldrich, cat. #I9030], 250 µL of 24.1 mM MTT [Sigma-Aldrich, cat. #M2128], 250 µL of 6.5 mM PMS [Sigma-Aldrich, cat. #P9625], and 2 mL of 1.6% hot agar [Sigma-Aldrich, cat. #A5306]; PMS is photosensitive and should only be added immediately before staining along with the agar [Hebert and Beaton, 1993]). Staining is usually sufficient for resolution of the alleles after 30 seconds to 1 minute. Recall that ADH is a dimeric protein, so three distinct bands corresponding to ADH-F/ADH-F, ADH-F/ADH-S, ADH-S/ADH-S, will be observed. Once stained, the agar overlay is washed off and individual flies (lanes on the gel) are scored for genotype (*Adh-F/Adh-F, Adh-F/Adh-S, Adh-S/Adh-S*; see Figure 1).

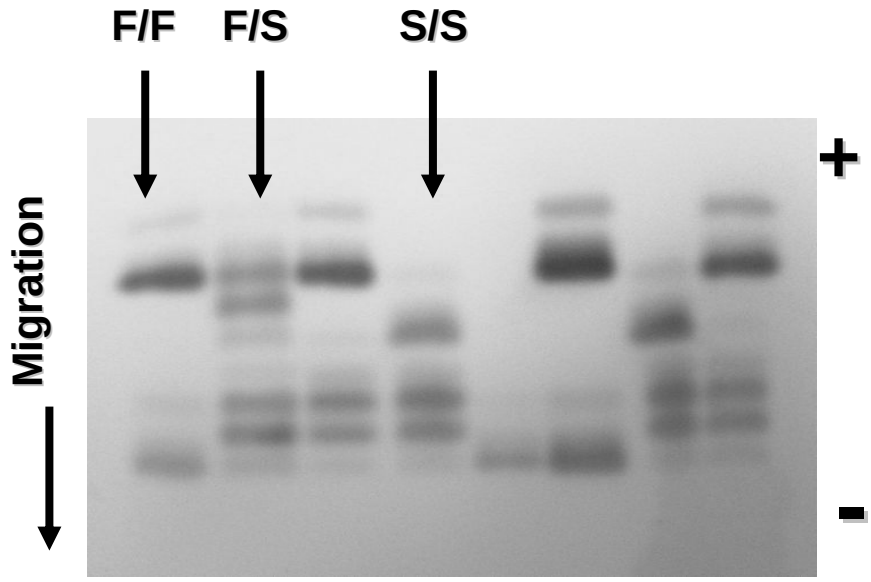

Figure 1. Cellulose acetate gel of 16 samples after electrophoresis of whole-fly homogenates and staining for ADH. Allozymes migrate towards the anode in which the ADH-S homozygotes migrate faster than ADH-F homozygotes (note that in this electrophoretic separation the "classical" Slow allele actually migrates faster than the "classical" Fast allele). Heterozygotes show multiple banding indicating that the fast and slow alleles can form all three dimer combinations.

Table 1. Chi-squared analysis of observed values for each genotype compared to the expected values and the Hardy-Weinberg allelic and genotype frequencies. Chi-squared value obtained can be compared to standard value tables to confirm a fit to the Hardy-Weinberg Equilibrium.

Genotype	Observed	Expected	$(O-E)^2/E$
Adh-S/Adh-S	19	14.628	1.306698
Adh-F/Adh-S	25	34.224	2.486038
Adh-F/Adh-F	25	20.148	1.168449
	Total= 69		X^2= 4.96

p = 0.460, q = 0.540, p^2 = 0.212, q^2 = 0.296, $2pq$ = 0.196

Table 2. One factor ANOVA of genotype vs. ADH enzyme activity. The analysis suggests that there are significant differences in ADH enzyme activity among the three genotypes.

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	2	17226.979	8613.49	50.9167
Error	66	11165.105	169.17	
C. Total	68	28392.085		

*Prob> F is <0.0001

Table 3. Tukey's HSD analysis examines and groups the data into blocks in which groups that do not share letters are significantly different. The analysis has revealed that there are significant differences among the 3 genotypes. The mean ADH enzyme activity and standard error are also given.

Level				Mean	Std Error
Adh-F/Adh-F	A			48.79264	2.601294
Adh-F/Adh-S		B		24.33155	2.838245
Adh-S/-Adh-S			C	11.58463	2.712037

Levels not connected by same letter are significantly different.

ADH Activity

The ADH enzyme activity for each fly is determined using the same whole-fly homogenates used in genotyping. ADH V_{max} is assayed by measuring the conversion rate of NAD^+ to NADH spectrophotometrically at 340 nm under saturated substrate conditions. 10 µL of homogenate is added to 100 µL of ADH assay solution (0.1 M Tris-HCl, pH 8.6, 4.0 mM NAD^+, 0.2 M isopropanol), mixed thoroughly for 30 seconds, and the absorbance at 340 nm (A_{340}) is measured every 9s for 3 min at 25°C. The slope of the increase in A_{340} gives a relative estimate of ADH V_{max} for each fly. The ADH activity assay can be performed on a 96 well plate and each sample can be replicated 2 or 3 times with the average activity used for data analysis.

Data Analysis

Fast and *slow Adh* allelic frequencies are measured and tested for Hardy-Weinberg Equilibrium. Since *D. melanogaster* is diploid and *Adh* is an autosomal locus, each fly contributes two alleles to the analysis. Figure 1 is an example of a cellulose acetate gel after electrophoresis and staining showing the migration of each genotype. For testing of Hardy-Weinberg Equilibrium, genotype frequencies are estimated as *Adh-S/Adh-S:* p^2, *Adh-F/Adh-S:* $2pq$, and *Adh-F/Adh-F:* q^2, where *fast* and *slow* allelic frequencies are represented by p and q, respectively. For example, in 2008 we assayed 69 flies from isofemale lines established in Sudbury, Ontario. Cellulose acetate electrophoresis indicated 19, 25, and 25 *Adh-S/Adh-S, Adh-F/Adh-S,* and *Adh-F/Adh-F* genotypes, respectively (Table 1), and allelic frequencies were, therefore, p = 0.460 and q = 0.540. It is possible to establish if the assayed population follows the Hardy-Weinberg Equilibrium by chi-squared analysis to compare observed (O) to expected (E) genotype numbers, where the expected genotype numbers are the respective frequencies (p^2, $2pq$, or q^2) multiplied to the total number of samples.

ADH enzyme activities are sorted by genotype and an Analysis of Variance (ANOVA) test and a Tukey's Honestly Significant Difference (HSD) test conducted to identify possible significant differences in enzyme activity between the 3 genotypes. Figure 2 shows results from the 2008 experiment showing substantial differences in ADH activity among the 3 genotypes: the flies

homozygous for the *fast* allele (the slower migrating allele) show the highest amount of activity and the heterozygous flies show intermediate activity. The ANOVA (Table 2) suggests significant differences amongst each group ($F_{2, 68} = 50.92$, $p < 0.0001$). Tukey's HSD analysis (Table 3) indicates that all three genotypic groups have distinct ADH activities. Both the ANOVA and Tukey's HSD analyses were conducted through JMP 7 statistical analysis software (SAS Institute Inc.), however, any statistical analysis software should suffice.

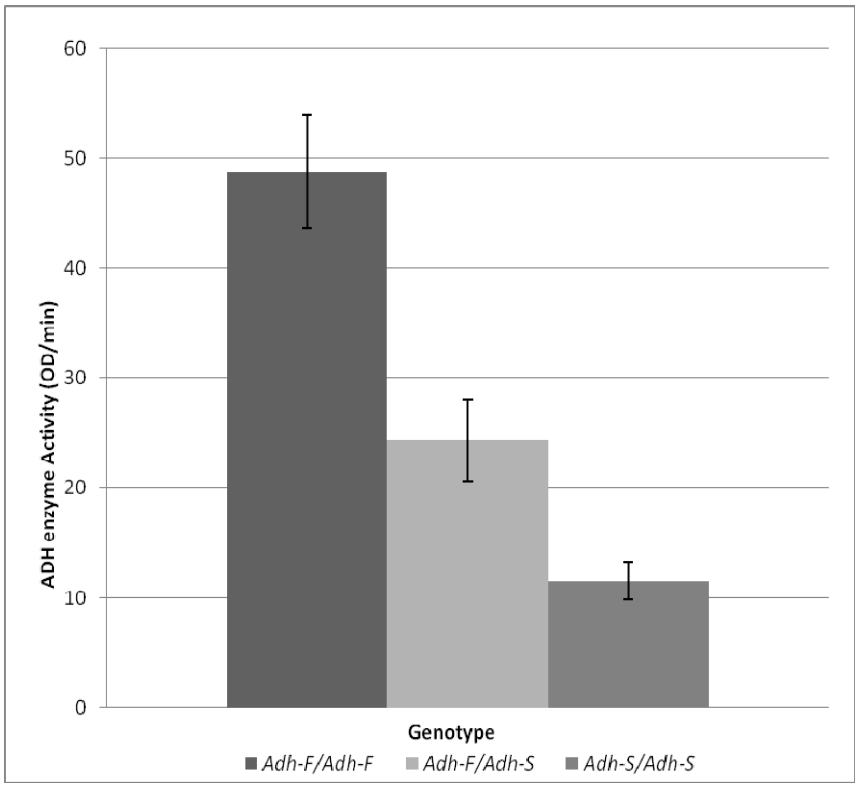

Figure 2. The average ADH enzyme activity for each genotype. Activity is highest for ADH-F homozygotes and lowest for ADH-S homozygotes. As in Figure 1, it is important to note that cellulose acetate electrophoresis reverses the migration rates of the two alleles and, therefore, the slower moving ADH-F homozygote will have the highest activity.

Conclusion

This teaching protocol offers students an excellent opportunity to gain hands-on experience with *Drosophila melanogaster,* a model species used in many current and classic genetic studies. Students learn the basics of genotype identification, enzyme kinetics, population genetics, and both genetic and biochemical data analysis. Furthermore, instructors are able to expand or shorten this protocol to fit their teaching requirements. An interesting expansion would be to collect local or endemic species of *Drosophila* and compare the *Adh* population genetics and enzyme activity to *D. melanogaster.* The experiments described here are cost effective, simple, and produce reliable results. Finally, in writing up the experiments, students have the opportunity to explore the enormous wealth of experimental work that has been devoted to this system over the past three-plus decades.

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