

**Absence of Dosage Compensation in the X-linked Acid Phosphatase Gene,
Ap-6, in *Drosophila pseudoobscura* and *D. miranda***

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Introduction

Drosophila pseudoobscura and *D. miranda* are two closely related sibling species (5). Interspecific hybrids can be produced in the laboratory between these species (2,3,16), however, hybrids have not been found in nature (4).

Drosophila miranda has three, instead of two, sex-determining chromosomes (2), namely X^1 , X^2 , and Y. The *D. miranda* females have $X^1X^2X^2$ plus three pairs of autosomes and the males have X^1X^2Y plus three pairs of autosomes. *Drosophila pseudoobscura* females have XX plus four pairs of autosomes, and the males have XY plus four pairs of autosomes. Dobzhansky and Tan (6) confirmed homologies between *D. miranda* and *D. pseudoobscura* chromosomes and noted the arrangements that had occurred. One homology demonstrated was between the *miranda* X^1 and *pseudoobscura* X.

In species with an XY, ZW, or XO method of sex determination, the homogametic sex has twice the number of X-linked gene copies as the heterogametic sex. However, the levels of many X-linked gene products are equivalent in males and females. In *Drosophila* both X chromosomes function in all the somatic cells of females (10,14). Equilization of X-linked gene product in *D. melanogaster* was first described in 1922 by Bridges (1) and termed "dosage compensation" by Muller in 1931 (17). Since then many genes on the X chromosome of *D. melanogaster* have been demonstrated to be dosage compensated (13). However, the molecular mechanisms of dosage compensation remain unknown (15).

There are a few reports of the lack of dosage compensation. A noncompensated wildtype X-linked gene in *D. melanogaster* codes for the alpha chain of larval serum protein-1 (19). The enzyme 6-phosphogluconate dehydrogenase (6-PGD) was shown to be Z-linked and not compensated in *Heliconius* females (9). In *D. melanogaster* 6-PGD is X-linked and is dosage compensated (11). My research indicates that the acid phosphatase-6 (*Ap-6*) gene is not dosage compensated in *D. pseudoobscura* or *D. miranda*. The *Ap-6* gene is X-linked in *D. pseudoobscura* (18) and due to homology is presumed to be X^1 -linked in *D. miranda*.

Methods and Materials

Species of *Drosophila* used to investigate the acid phosphatase enzymes (AP-6, AP-3, AP-1, and AP-Trace) were three *D. pseudoobscura* stocks (Riv100 from Riverside, California, WWA-6, and WWA-7) and a *D. miranda* stock (S204 from Sisters, Oregon). The Riv100 and S204 stocks were obtained from the laboratory of Satya Prakash, University of Rochester, New York. The WWA-6 and WWA-7 stocks were from the Species Resource Center, Bowling Green State University, Bowling Green, Ohio. All fly cultures were reared at 18°C on an instant potato-yeast-acetic acid medium.

Reciprocal interspecific crosses were made between *D. pseudoobscura* Riv100 and *D. miranda* S204 for investigation of acid phosphatase gene expression. Newly eclosed flies were collected from the maintenance stocks, aged separately by sex for 5-7 days, and then mated to produce hybrid offspring. The viable F₁ hybrids produced from

the reciprocal crosses have the genotype, A3/X²,X/X¹, expressed in terms of the sex chromosomes and homologous chromosomes of the two species. The *D. pseudoobscura* autosome 3 (A3) is homologous to the *D. miranda* X² chromosome and the *D. pseudoobscura* X chromosome is homologous to the *D. miranda* X¹ chromosome. Other possible genotypes from the interspecific crosses either had low viability (A3/X²,X and X²/A3,X¹/Y) or did not eclose (A3,X/Y and A3,X/X¹Y). Parental and F₁ flies, eclosing over an eight hour period (6:00 a.m.-2:00 p.m.), were collected daily and aged from one to 40 days at 18°C in groups of 50 flies or less per vial. Whole flies were homogenized at 10:00 a.m. on the same day that electrophoresis was conducted. Therefore, the homogenates were prepared from flies 0-40 days + - four hours.

Intraspecific reciprocal crosses were made between the WWA-6 and WWA-7 strains of *D. pseudoobscura*. These strains were electrophoretically polymorphic for the *Ap-6* locus and were chosen to verify X-linkage of the *Ap-6* locus.

Flies were etherized and then weighed in groups of 10-20 individuals to an accuracy of 0.1 mg. Whole flies, placed in 250 µl Beckman or 1.5 ml Eppendorf microcentrifuge tubes in an ice bath, were ground at a concentration of one fly per 4 or 5 µl of Tris-Borate (TB) grinding solution. This solution consisted of 100 ml 0.1 M tris(hydroxymethyl)aminomethane-borate (Tris-Borate) buffer pH 8.9-9.1, 6 gm sucrose and sufficient bromphenol blue to act as a tracking dye (8). The resulting homogenates were centrifuged in an Eppendorf 5412 Microcentrifuge at 15,600 x g for 1.5 minutes. After centrifugation, samples were stored on ice until they were layered onto acrylamide gels.

A vertical gel electrophoresis apparatus (EC490 from E-C Apparatus Corporation) was used for separation of the enzymes in the fly homogenate supernatant. Six percent gels, consisting of 95 percent acrylamide (Sigma) and five percent N,N1-methylene-bis-acrylamide (Sigma), were prepared by dissolving 19.95 gm of acrylamide and 1.05 gm of bis-acrylamide in sufficient 0.1 M Tris-Borate buffer (pH 8.9-9.1) to attain a volume of 350 ml. After filtration, polymerization was achieved with the addition of 0.7 ml tetramethyleneethylenediamine (TEMED) and 1.75 ml ten percent ammonium persulfate per 350 ml gel solution. Tris-Borate (0.1 M, pH 8.9-9.1) was used in the gel box.

Prior to electrophoresis, gels were cooled to -5°C and maintained at that temperature throughout electrophoresis. Five µl of supernatant from the centrifuged fly homogenates were layered into the 24 gel pockets and electrophoresis was conducted at a constant 350 volts with each gel box drawing approximately 100 milliamperes for 2.5 hours.

Following electrophoresis, the gels were stained for acid phosphatase by immediately immersing them in a staining solution. This solution consisted of a buffer mixture (90 ml 0.1 M sodium acetate buffer pH 5.0, 10 ml 20 percent NaCl, 5 ml 0.5 M MgCl₂, and 0.5 ml 10 percent MnCl₂) to which was added immediately prior to gel immersion, 100 mg Fast Blue (BB-salt), 500 mg polyvinylpyrrolidone, and 100 mg α-naphthyl acid phosphate (Sigma). Since this stain is light sensitive, the mixing beaker and gel developing dish were shielded from light.

Acid phosphatase bands began to appear within two hours of developing, but for maximum visibility, gels were stained overnight. Electropherograms were destained, fixed, and stored at room temperature in a solution of five parts methanol, five parts distilled water, and one part acetic acid prior to densitometric analysis.

Electropherograms were densitometrically scanned using an EC910 Densitometer (E-C Apparatus Corporation) for documentation of the amounts of acid phosphatase in the electrophoretic separations. The gel was positioned to allow the light beam to pass successively through each of the 3 to 5 acid phosphatase bands per pocket lane.

The transmitted light intensity, registered by a recorder, resulted in a densitogram for each separate homogenate sample (7).

The area under each peak of the densitogram was determined. This area is a measure of the enzyme activity that was present in each electrophoretic separation of the acid phosphatases on the electropherograms. X-y coordinates, taken from the densitographic curves were transmitted to the DECsystem 10 computer (Digital Equipment Corporation, Maynard, Mass.) and analyzed using the MLAB program. MLAB was selected for its curve fitting program which adjusts the parameters of a model function to minimize the sum of squared errors (12). Curve analysis (or determination of area under each peak of the densitogram) was based on a mathematical model of overlapping normal curves.

Results and Discussion

The interspecific cross of *D. pseudoobscura* females and *D. miranda* males (Cross A) and the reciprocal (Cross B) yielded vigorous sterile F₁ females which were collected for further analysis. These hybrid females have the genotype A₃/X², X/X' in terms of sex chromosomes. The *Ap-6* X-linkage in *D. pseudoobscura* was verified as follows: preliminary studies of WWA-6 and WWA-7 *D. pseudoobscura* strains demonstrated electrophoretically polymorphic *Ap-6* loci. Strain WWA-6 has a double electrophoretic AP-6 band and strain WWA-7 has a single electrophoretic AP-6 band (Figure 1). The F₁ males from the mating of WWA-6 males and WWA-7 females elec-



FIGURE 1. Electropherogram demonstrating X-linkage of the *Ap-6* locus in *D. pseudoobscura*. WWA-6 and WWA-7 are the *D. pseudoobscura* strains crossed. F₁ from a particular cross are placed between the parental flies. Pockets 1-6 contain flies of the cross WWA-6♂ x WWA-7♀. Pockets 7-11 contain flies of the cross WWA-7♂ x WWA-6♀. Arrows indicate F₁ male hybrids.

trophoretically demonstrated only the single band due to the *Ap-6* locus of the WWA-7 female. In the reciprocal mating of WWA-7 males and WWA-6 females, the F_1 males show the double electrophoretic AP-6 band of the WWA-6 females (Figure 1). Therefore, the *Ap-6* locus is on the X chromosome of *D. pseudoobscura*. In *D. miranda* *Ap-6* is presumed to be on the X' chromosome since X and X' are reported to be predominantly homologous. The hybrid females are, therefore, carrying the *pseudoobscura Ap-6* on the X chromosome and the *miranda Ap-6* on X'.

Electrophoresis detected four separate adult acid phosphatases in the *Drosophila* species studied: acid phosphatase-6 (AP-6), acid phosphatase-3 (AP-3), acid phosphatase-1 (AP-1) and acid phosphatase-trace (AP-Tr). The *Ap-3* locus was electrophoretically polymorphic in the strains, Riv100 and S204, used. Therefore, three mobility bands appeared for AP-3 in the female hybrids (Figure 2).

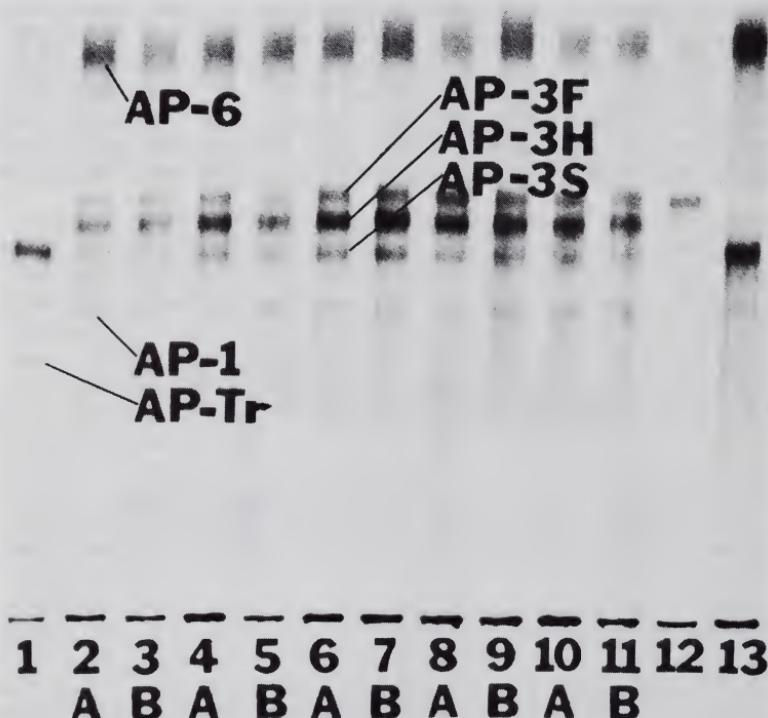


FIGURE 2. Electropherogram of *D. pseudoobscura*, *D. miranda*, and interspecific F_1 hybrids. The acid phosphatases are AP-6, AP-3 Fast (AP-3F), AP-3 Heterodimer (AP-3H), AP-3 Slow (AP-3S), AP-1, and AP-Trace (AP-Tr). Lane 1 is a *D. pseudoobscura* male, lane 12 a *D. miranda* female, lane 13 a *D. pseudoobscura* female and lanes 2-11 are F_1 hybrid females labelled A and B corresponding to crosses A and B.

Densitometry was used to determine the enzyme activity of the acid phosphatases studied. Densitometric measurement of the enzymes in each pocket lane of an electropherogram produced a densitogram. Measurement of acid phosphatases in F_1 hybrid

females generated single peaked curves for AP-6 and AP-1 (Figure 3). The tri-peaked curve for AP-3 corresponds to the fast, intermediate, and slow bands of AP-3 in the hybrid (Figure 3).

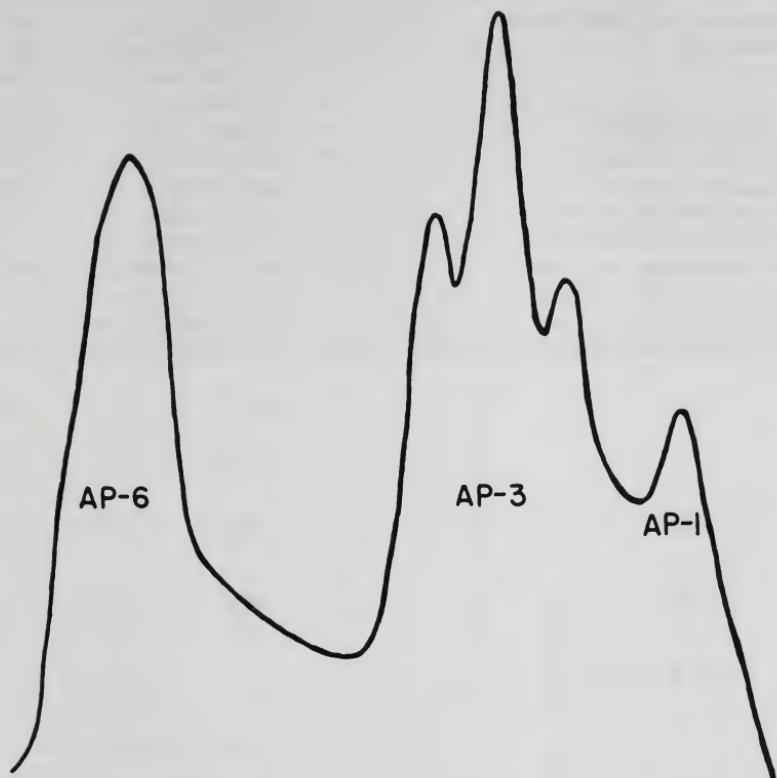


FIGURE 3. Densitogram of a *D. pseudoobscura* and *D. miranda* interspecific cross B F₁ hybrid female. The amounts of acid phosphatases, AP-6, AP-3, and Ap-1, present in the F₁ hybrid correspond to the areas under the curve.

Computer analysis of densitograms yielded a curve fit and a computation of area under each peak including those curves which overlapped. Mean proportions of AP-6 (AP-6 per total acid phosphatase present per sample) were also generated by the computer program (Table 1). These mean proportions allowed for gel to gel comparisons.

Variations in amounts of AP-6 occurred between and within species but not between hybrids (Table 1). Analysis of variance indicated that the level of AP-6 was significantly different ($p=0.000$) between the two species. *D. pseudoobscura* had a higher level of AP-6 than *D. miranda* (Table 1, Figure 4). However, there was no significant difference ($p>0.842$) between amounts of AP-6 present in the interspecific hybrids. Significant variation ($p=0.000$) in AP-6 level occurred between parental males and females. In both species the females had a greater amount of AP-6 in terms of proportion of enzyme as did the males. AP-6 analysis of variance revealed no significant interaction ($p=0.453$) where species and sex were the factors analyzed. This implied that the relationship of AP-6 levels of males to females was the same in the

TABLE 1. Mean proportions of acid phosphatase-6 activity per total AP measured in *D. pseudoobscura*, *D. miranda*, and interspecific F₁ hybrid females and calculated mean level activity per sex chromosome. Means are based on *n* samples per source of enzyme. S.D.=Standard Deviation.

Source of Enzyme	<i>n</i>	Mean AP-6 Activity	S.D.
<i>D. pseudoobscura</i> females (XX)	33	0.587	0.176
<i>D. pseudoobscura</i> males (X)	37	0.379	0.251
Mean level per X chromosome	—	0.322	-----
<i>D. miranda</i> females (X'X')	29	0.335	0.164
<i>D. miranda</i> males (X')	27	0.143	0.100
Mean level per X' chromosome	—	0.159	-----
Cross A hybrid (XX')	41	0.473	0.126
Cross B hybrid (XX')	30	0.477	0.143
Predicted hybrid level	—	0.481	-----

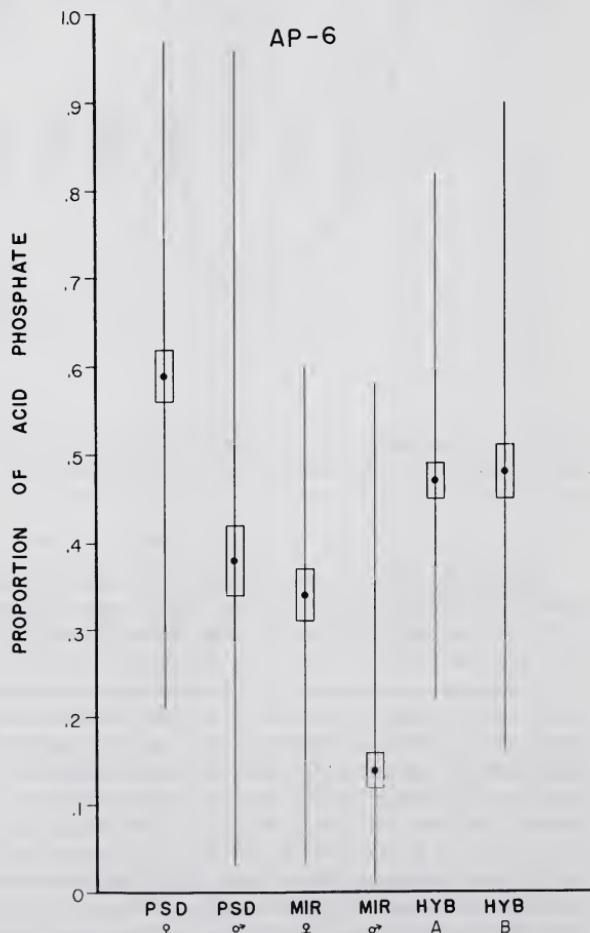
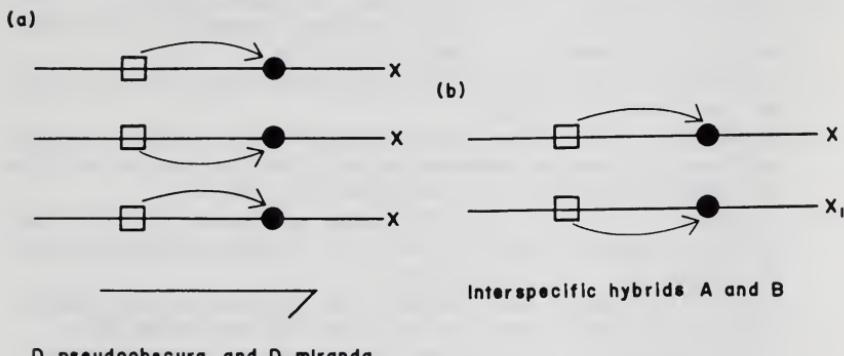


FIGURE 4. The range and mean of proportions of acid phosphatase-6 in *D. pseudoobscura*, *D. miranda*, and interspecific hybrid F₁ females. Each vertical line indicates the range, the dot the mean, and the rectangle the standard error of the mean of acid phosphatase-6 for each species' males and females and hybrids A and B.

two species. Combining the relative proportions of AP-6 produced by the two X chromosomes in *D. pseudoobscura* females (0.587) and the one X chromosome in *D. pseudoobscura* males (0.379), a total level of 0.966 was obtained (Table 1). This indicated the production of a mean level of 0.322 per *D. pseudoobscura* chromosome. Therefore, the predicted relative proportion in *D. pseudoobscura* females would be 0.644. The observed level in *D. pseudoobscura* females was 0.587. In *D. pseudoobscura* males, the predicted relative proportion of AP-6 would be 0.322 and the observed level was 0.379. Furthermore, in *D. miranda* the *Ap-6* locus was assumed to be on the X' chromosome because a large proportion of the X' is homologous to the *D. pseudoobscura* X chromosome (6). The predicted relative proportion of AP-6 produced by each X' chromosome would be 0.159 (Table 1). The observed level of AP-6 in *D. miranda* females with two X' chromosomes was 0.345 and the observed level of AP-6 in *D. miranda* males with one X' chromosome was 0.143. T-tests validated the hypothesis that the relative proportion of AP-6 in females was equal to two times the relative proportion of AP-6 in males ($0.4 > p > 0.05$) in both species.

A *cis*-acting model of *Ap-6* gene regulation is proposed for *D. pseudoobscura* and *D. miranda* (Figure 5). The males in both species produced one-half the amount



D. pseudoobscura and *D. miranda*
♀ and ♂

FIGURE 5. Regulatory models of AP-6 production in *D. pseudoobscura*, *D. miranda*, and interspecific hybrids A and B. a) *Cis*-acting regulation of the *Ap-6* loci in *D. pseudoobscura* and *D. miranda* males and females. b) *Cis*-acting regulation of the *Ap-6* loci in interspecific hybrids A and B.

◻-regulatory locus
●-structural *Ap-6* locus

of gene product present in the females. Both *cis*-acting modifiers are proposed to affect only their adjacent *Ap-6* loci in the hybrid system (Figure 5). The *D. pseudoobscura* X chromosome hypothetically should produce a relative activity of 0.322 and the *D. miranda* X' hypothetically should produce a relative activity of 0.159. Therefore, the predicted hybrid activity would be 0.481 (Table 1). This value was not significantly different ($0.7 > p > 0.6$) from the measured activity of 0.473 in hybrid A or the measured activity of 0.477 in hybrid B (Table 1). Further elucidation of *Ap-6* gene regulation could be obtained with a study utilizing AP-6 electrophoretic mobility variants so that enzyme activities could be separately measured in the hybrid. Also, additional studies utilizing heads or thoraces for evaluation of AP-6 activity would rule out the possibility of high concentrations of that enzyme in female sex organs which could account for the difference in enzyme quantity between the sexes. Experiments are planned to

discriminate the occurrence of AP-6 in various organs and body parts. Studies to be reported in coming publications on other acid phosphatases in *D. pseudoobscura* and *D. miranda* should further clarify *Ap-6* gene activity.

In conclusion, the proportions of AP-6 measured in the parental species and inter-specific hybrids substantiate lack of dosage compensation of the *Ap-6* locus in *D. pseudoobscura* and *D. miranda*. The integrity of gene expression in the X and X' chromosomes is maintained in the hybrid system, therefore, supporting a *cis*-acting model of gene regulation.

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