ENZYMES AND REPRODUCTION IN NATURAL POPULATIONS OF *DROSOPHILA EURONOTUS*[1]

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ABSTRACT

Populations of *Drosophila euronotus*, one from southern Louisiana (3 samples), and one from Missouri (2 samples), were classified for allele frequencies at alkaline phosphatase (APH) and acid phosphatase (ACPH) loci. The two populations differed consistently in allele frequencies at both loci. The APH locus is on the inversion-free *X* chromosome; the chromosomal locus of the autosomal ACPH is unknown, and could involve inversion polymorphism. Wild females from Missouri and Louisiana populations heterozygous at the APH locus carried more sperm at capture than did the corresponding homozygotes. This heterotic association was significant for the combined samples, and whether it was the result of heterosis at the enzyme locus studied, or due to geographically widespread close linkage with other heterotic loci, it should help to maintain heterozygosity at the APH locus. In a Louisiana collection which included large numbers of sperm-free females, simultaneous homozygosity at both enzyme loci was significantly associated with lack of sperm. It is suggested that the latter association is the result of young heterozygous females achieving sexual maturity earlier than do the double homozygotes. The average effective sperm load for 225 wild females was only 29.4, suggesting the necessity for frequent repeat-mating in nature to maintain female fertility. A comparison of the sex-linked APH genotypes of wild females with those of their daughters indicated that among 295 wild-inseminated females from five populations, 35% had mated more than once, and of this 35%, six females had mated at least three times. Because of ascertainment difficulties, it is clear that the true frequency of multiple-mating in nature must have been much higher than the observed 35%. Laboratory studies indicate that multiple-mating in this species does not involve sperm displacement, possibly due to the small number of sperms transmitted per mating, and the fact that the sperm receptacles are only partially filled by a given mating.

DARWINIAN selection in natural populations is in essence the differential reproduction of genotypes, involving two major components, viability and fertility. As ANDERSON and WATANABE (1974) point out, viability has received the most attention, both theoretically and experimentally, possibly because genetic differences in viability may sometimes be detected by distortions in the Hardy-Weinberg frequencies, while differences in fertility cause little or no such distortion, and thus are harder to demonstrate.

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The fertility component of selection involves not only production of gametes, fertilization and care of eggs or young, but also mating patterns, i.e., panmixis or its absence, minority effect (EHRMAN 1966), and the frequency and consequences of multiple matings. Some of these components of fertility are suceptible to direct study in natural populations, even in species which are poorly understood ecologically For example, in natural populations of *Drosophila melanogaster* STALKER (1976) has demonstrated non-random mating and reproductive superiority (probably in mating ability) in females heterozygous for inversions.

The present paper describes further studies on reproduction in natural populations, and its relation to enzyme genotypes.

MATERIALS AND METHODS

Enzyme variability was studied in natural populations of *Drosophila euronotus*, a species known primarily from the southeastern United States, especially from deciduous woods; it has been collected from Florida to eastern Texas in the south, and from Virginia to Iowa in the north. This species was chosen because its chromosomal variability is well understood (STALKER 1964); it frequently exists in large populations, especially in the south; it is easily bred in the laboratory; and the large adult body size facilitates enzyme determinations.

Three enzyme loci were studied; one sex-linked, alkaline phosphatase (APH), and two autosomal, acid phosphatase (ACPH) and esterase. KOJIMA, GILLESPLE and TOBARI (1970) have included esterases and phosphatases in that group of highly polymorphic enzymes which utilize external substrates. The esterase locus was soon determined to be unsuitable for further work since it had a very large number of alleles, producing electrophoretic bands which ran so close together as to make identification impossible without extensive retests of each wild fly or its progeny. The two phosphatase loci, on the other hand, showed smaller numbers of distinguishable alleles, and these were readily identified in starch gels. The particular autosome carrying the ACPH locus was not identified, and indeed, might be the second chromosome with its abundant inversion polymorphism. The sex-linked APH locus was clearly not involved with inversions; samples of *X* chromosomes taken from the two collecting areas (77 from Missouri, 83 from Louisiana) showed only Standard gene sequences. In *D. euronotus X* chromosome inversions are known only from peninsular Florida (STALKER 1964 and unpublished).

Wild flies were collected from fermenting banana bait, sorted and stored in a portable icebox until they reached the laboratory, and then either frozen directly, or in the case of many of the wild females, allowed to produce fertilized eggs until sperm exhaustion, and then frozen. The frozen adults were stored at —20° in small glass vials sealed with Parafilm. Tests showed that neither the cold storage for periods of up to a year, nor aging of adults in the laboratory for up to four weeks had any detectable effect on the outcome of the enzyme determinations.

The F_1 progeny of wild-inseminated females were reared to emergence, aged as adults for 4–12 days, and then frozen. Such progeny were used for estimates of the effective number and types of sperm carried by the wild females. Phosphatase banding patterns were determined in wild flies of both sexes, and in six to twelve F_1 progeny from each wild female. Wild females which were uninseminated at capture were pair-mated to wild males from the same collection, and enzyme determinations made on both parents and progeny in order to determine the genetic basis of the phosphatase variability. Numerous progeny tests of this sort indicated that both the APH and ACPH molecules that were studied were dimers, with homozygotes showing a single band, and heterozygotes showing three bands, with the middle "hybrid" band heavier than the other two.

Electrophoresis Methods: Proteins were separated by horizontal starch gel electrophoresis, using Sigma starch (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 11 (w/v) in Poulik's buffer at pH 8.7. The NaOH — H_3BO_3 tray buffer was adjusted to pH 8.4. Gels were run for approximately 5 hours at 90 ma. in a 4° cold box. All staining was done with Fast Blue

RR at 37°; the bottom gel slice was stained at pH 4.4. for ACPH, and the middle slice at pH 7.4 for APH. The stained gels were fixed in acetic-ethanol, wrapped in Saran Wrap, and stored at —20°.

Fresh or frozen flies were ground in distilled water in wells in a chilled plexiglass grinding block using an electric drill equipped with a plexiglass rod. The ground samples were soaked up by a strip of filter paper and inserted in the gel origin. In some cases, after the flies were ground, the grinding wells were sealed with Scotch Brand Magic Tape, and the blocks and samples stored overnight at —20° for use the following morning.

Each starch gel was loaded with approximately equal numbers of experimental and control samples; in most cases this permitted immediate identification of the experimental bands. In cases of uncertainty further tests were run on F_1 progeny, using a more selective array of standards.

Population samples were taken from two localities; one of these was in Webster Groves, Missouri, a suburb of St. Louis. The immediate collecting site supported 32 species of Drosophilidae, and hence had some of the ecological characteristics of the local deciduous woods. The Missouri collections are listed in the tables as MO-70 (Aug.–Sept., 1970) and MO-71 (June, 1971). The second locality was in Fontainebleau State Park in southern Louisiana, on the north shore of Lake Ponchartrain. In this park the flies were trapped from a roughly rectangular 20 × 120 meter site, in which the vegetation showed a rather abrupt change from a very dense deciduous thicket (including Hyrax, Tupelo, Sweetgum, Poison Ivy and Post Oak) at one end to an open forest of Yellow Pine, Sassafras and Palmetto at the other. The Louisiana study was terminated in 1972 when the Drosophila in the trapping site were essentially wiped out, apparently by drought and/or insecticide drift from a picnic area 500 meters distant. The Louisiana collections are listed in the tables as LA-70 (Oct., 1970), LA-71j and LA-71o (June and Oct., 1971, respectively).

RESULTS

Tables 1 and 2 give the observed gene frequencies for all major collections, based on lumped data from wild-caught males and females (but not from their F_1 progeny). In Table 3 are listed the significant gene frequency differences between areas and between sequential collections within areas. Table 3 may be summarized as follows:

Autosomal ACPH locus, (Table 3A): The Louisiana population differed from that of Missouri in having significantly higher frequencies of alleles *C* and *D*, and lower frequencies of allele *E*. In addition, the frequency of the allele *D* rose significantly in the 1970–71 period in both Missouri and Louisiana.

TABLE 1

Percentage frequencies of alleles at autosomal acid-phosphatase locus in wild adult Drosophila euronotus

Population	N	*C*	*D*	*E*	*H*	*I*	*O*	*M*	*W*	*Null*
Missouri, MO-70	85	50.59	1.18	47.06	—	—	1.18	—	—	—
Missouri, MO-71	50	48.00	20.00	32.00	—	—	—	—	—	—
Louisiana, LA-70	154	68.83	20.78	9.09	0.65	—	0.65	—	—	—
Louisiana, LA-71j	193	59.59	29.53	8.29	1.04	0.52	0.52	—	—	0.52
Louisiana, LA-71o	537	71.14	17.13	8.19	1.30	0.19	1.12	0.56	0.37	—

N = number of alleles sampled from each population.

TABLE 2

Percentage frequencies of alleles at sex-linked alkaline phosphatase locus in wild adult Drosophila euronotus

Population	N	*A*	*B*	*F*	*G*	*A″*
Missouri, MO-70	81	48.15	37.04	8.64	4.94	1.23
Missouri, MO-71	48	45.83	33.33	20.83	—	—
Louisiana, LA-70	131	58.78	24.43	14.50	1.53	0.76
Louisiana, LA-71j	176	60.80	22.16	14.20	2.84	—
Louisiana, LA-71o	442	56.11	25.34	16.52	0.68	1.36

N = number of alleles sampled from each population.

Sex-linked APH locus, (Table 3B): Louisiana collections showed consistent and significantly higher frequencies of allele *A*, and lower frequencies of allele *B* than did the Missouri collections. In addition, the *F* allele frequency rose significantly in the 1970–71 period in Missouri.

A few cases of significant gene frequency differences were observed between samples collected from various parts of the Louisiana site, but these differences were not maintained in successive years, and may have been the result of drift.

No significant gene frequency differences were observed between wild males and females, nor between wild adults and the F_1 laboratory-produced progeny. This latter result suggests that the genetically different types of wild males were proportionately represented in the sperm carried by the females, and hence the various male genotypes were equal or very similar in their reproductive efficiency.

No significant deviations from Hardy-Weinberg frequencies were found in wild males, in wild females, nor in their F_1 progeny. The allele at the APH locus appeared to be associated at random with those at the ACPH locus in wild adults and in their F_1 progeny. Finally, analysis of a single sperm from each wild-

TABLE 3A

Significant differences in autosomal acid phosphatase allele frequencies among populations and samples

Allele	Frequency difference	χ^2	d.f.	P
C	LA-70, LA-71o > LA-71j	8.48	1	0.004
	LA-70, LA-71o > MO-70	14.06	1	0.0002
	LA-70, LA-71o > MO-71	11.18	1	0.0008
D	LA-71j > LA-70, LA-71o	12.44	1	0.0004
	MO-71 > MO-70	12.50	1	0.0004
	LA-70, LA-71o > MO-70	15.75	1	0.0001
	LA-71j > MO-70	28.74	1	<0.0001
E	MO-70 > LA-70, LA-71o	102.55	1	<0.0001
	MO-70 > LA-71j	55.14	1	<0.0001
	MO-71 > LA-70, LA-71o	28.91	1	<0.0001
	MO-71 > LA-71j	19.52	1	<0.0001

TABLE 3B

Significant differences in sex-linked alkaline phosphatase allele frequencies among populations and samples

Allele	Frequency difference	χ^2	d.f.	P
A	LA-70, LA-71j, LA-71o > MO-70, MO-71	4.83	1	0.028
B	MO-70, MO-71 > LA-70, LA-71j, LA-71o	7.19	1	0.007
F	MO-71 > MO-70	3.92	1	0.048

inseminated female homozygous at a particular locus indicated that the sperms carried by females are a random sample from the adult male population, thus giving no evidence for assortative mating of the kind found in *D. melanogaster* (STALKER 1976).

Phosphatase genotypes and reproductive potential of wild females. Wild females which consistently carry many sperm in their receptacles should have a greater reproductive potential than those that carry few sperm, since the posssession of well-stocked sperm receptacles would not only permit continued reproduction in the absence of males, but would stimulate oviposition.

Absence or scarcity of sperm in wild females could be explained by: (1) immaturity, with the females still virgin at capture; (2) ineffective preservation of sperm in the sperm receptacles; (3) lack of success in finding mates. Any of the three conditions above might be associated with relatively unfavorable genotypes, since maturing rate of the imagines, efficiency of the sperm receptacles, and mating success should all have a genetic component. Tests of *D. melanogaster* have shown that in some natural populations females heterozygous for autosomal inversions do carry significantly more sperm at capture than do the homozygotes (STALKER 1976). To test the possibility of a similar heterotic association with sperm load in *D. euronotus* wild-caught females were permitted to oviposit in the laboratory until they had exhausted their sperm, or had shown by the production of infertile eggs, that they were not carrying sperm at capture, after which the females were analyzed for phosphatase loci, and the maternal genotypes compared with the total number of offspring produced, the latter number being used as an estimate of effective sperm load at capture. Complete progeny counts were made for wild females from three populations: MO-71 from Missouri, and LA-71j and LA-71o from Louisiana.

No significant differences in number of progeny were found to be associated with any specific APH or ACPH alleles. Further, females heterozygous at the ACPH locus did not produce significantly more progeny than did the ACPH homozygotes. However in the case of the sex-linked APH locus, heterozygous females produced more offspring than did homozygotes. The data are summarized in Table 4; the one-tailed P values in the body of the table were derived from Mann-Whitney U-tests. It will be noted that the average number of progeny from heterozygotes was larger than that from homozygotes in all three populations, and that this increased progeny number approached significance in the first two populations. The natural logs of the three P values were summed, with the quantity

TABLE 4

Total progeny produced by wild-inseminated females prior to sperm exhaustion. Comparison of homozygotes and heterozygotes at the sex-linked alkaline phosphatase locus

Population	Homozygous N	Homozygous Ave. Progeny	Heterozygous N	Heterozygous Ave. Progeny	Mann-Whitney z	Mann-Whitney P^*	ln P
Missouri, MO-71	9	28.67	12	50.50	1.564	0.0589	—2.8319
Louisiana, LA-71j	30	25.90	43	32.58	1.665	0.0483	—3.0303
Louisiana, LA-71o	47	24.85	81	28.12	0.911	0.1814	—1.7071
							—7.5693
	$-2(\Sigma \ln P) = 15.1386$		d.f. $= 6$		Combined $P = 0.019$		

* P values are one-tailed.

$-2(\Sigma \ln P) = 15.14$ distributed as X^2 with 6 degrees of freedom, yielding a highly significant P value of 0.019 for the three populations combined, (see SOKAL and ROHLF 1969). This result indicates a significant heterozygous advantage as far as female sperm load is concerned, and is in general agreement with the *D. melanogaster* results described above.

The reason for the observed association of genotype and sperm load in *D. euronotus* is unknown. It could be the result of more frequent mating of the heterozygous females, their better preservation of sperm once acquired, or more effective matings (with more sperm per ejaculate). It is extremely unlikely that the effect could be attributed to wild heterozygous females refraining from ovipositing, and thus failing to use up their sperm supplies, since in this species, as in many others, initiation and rate of oviposition is positively correlated with sperm load. For example, in the LA-71o population sample, inseminated females beginning oviposition during the first three days after being set in the laboratory produced an average of 31.4 ± 1.84 progeny; those beginning oviposition during the 4th to 6th days produced an average of 20.3 ± 2.68 offspring while those beginning oviposition at day 7 or later produced only 12.1 ± 2.00 offspring. The average delay in onset of oviposition for the 128 inseminated females was 3.8 days, while for 48 non-inseminated females from the same population the average delay was 15.9 days.

It is of interest that it is the sex-linked APH locus (that known to be associated with no inversions), which shows a significant relationship with sperm load. This relationship could be explained either by heterosis at the APH locus itself, or by a geographically widespread association with other closely linked loci which are themselves heterotic. In any case, the observed heterotic effect should act as a balancing mechanism for retention of polymorphism at the APH locus.

Only females which carried some sperm at capture were included in Table 4. In the first two population samples nearly all of the wild-caught females did in fact carry sperm, however in the Louisiana population LA-71o, 48 of the females were without sperm at capture. The data on the relationship between genotype and sperm presence are given in Table 5.

TABLE 5

Homozygosity at APH and ACPH phosphatase loci in females with sperm, and those without sperm at time of capture. From Louisiana LA-71o population. Expected numbers are italicized

		APH Homo.	APH Hetero.	ACPH Homo.	ACPH Hetero.	APH and ACPH loci together Both Homo.	APH and ACPH loci together One or both Hetero.
With sperm	Obs.	47	81	72	56	26	102
	Exp.	*51.64*	*76.36*	*77.09*	*50.91*	*32.00*	*96.00*
Without sperm	Obs.	24	24	34	14	18	30
	Exp.	*19.36*	*28.64*	*28.91*	*19.09*	*12.00*	*36.00*
Contingency χ^2		2.559		3.099		5.500	
One-tailed P value		0.055		0.039		0.0095	

It will be noted that for both the APH and ACPH loci there is an observed excess of homozygous females without sperm, and heterozygotes with sperm at capture. These deviations from expectation approach significance for the APH locus ($P = 0.055$) and are significant for the ACPH locus ($P = 0.039$). A consideration of both loci simultaneously in the third section of Table 5 leads to a highly significant association ($P = 0.0095$) between heterozygosity and presence of sperm; females homozygous at both loci show an excess of individuals *without* sperm, those heterozygous at one or both loci show a corresponding excess of individuals *with* sperm. It should be pointed out that the significant associations with the autosomal ACPH locus may involve inversion heterozygosity.

The sperm load data for the three population samples in Table 4 are given in more detail in Table 6. The three samples differ markedly in the frequencies of sperm-free females. It is suggested that the 48 sperm-free females in LA-71o represent a recent mass emergence with their higher homozygote frequency the result of rapid maturity and insemination of some of the young heterozygotes. It might be objected that the higher frequency of homozygotes among these females

TABLE 6

Sperm loads among wild females of D. euronotus

Population sample	No. insem.	Average sperm load	Distribution of inseminated ♀♀ by sperm load 1–20	21–40	41–60	61–80	81–90	No. ♀♀ without sperm*
MO-71	21	41.1 ± 6.2	7	4	3	5	2	4
			33.3	*19.0*	*14.3*	*23.8*	*9.5*	
LA-71j	76†	30.4 ± 2.4	31	26	12	5	2	1
			40.8	*34.2*	*15.8*	*6.6*	*2.6*	
LA-71o	128	26.9 ± 1.5	53	44	26	5	0	48
			41.4	*34.4*	*20.3*	*3.9*	*0*	

* Not included in percentage calculations.
† Total includes 3 females incompletely genotyped.
Numbers in italics are percentages.

could be a chance phenomenon, rather than the result of selection, with most of the sperm-free females having been produced by a small number of breeding adults. This seems unlikely, since these 48 females were collected over the entire nine-day collecting period, and were evenly distributed throughout the collecting area.

Finally, it seems improbable that many of these 48 females were mature, had mated, and then had exhaused their sperm supply, indicating a general sperm shortage in the population. If this explanation applied to LA-71o (and it did not apply to either of the other two samples), then it would be expected that large numbers of females would have been captured with nearly exhausted sperm loads, e.g. in the 1–20 range. In fact, the sperm load distribution among females was practically identical for LA-71j and LA-71o for both the 1–20 and the 21–40 load ranges, thus lending no support to the idea of a general sperm shortage in the population during the LA-71o collection period.

The average sperm load (progeny number) for the 225 wild-caught *D. euronotus* females in Table 6 is only 29.4 per female. This figure contrasts sharply with an average progeny number of 180.2 for 305 wild-caught females of the sibling species, *D. paramelanica* (STALKER 1961 and unpublished), and in *D. melanogaster* with averages of 357.2 for 99 wild females from Missouri and 226.8 for 41 wild females from Mississippi (STALKER 1976).

D. euronotus females have shown similarly modest sperm storage under laboratory conditions. BARBARA GALLER, in preliminary studies on the effects of multiple matings in this species, mated each of 37 homozygous autosomal recessive frosty females to both a virgin wild-type male, and a virgin frosty male; the two matings were 48 hours apart, in 20 cases the first mate was wild-type, in 17 cases it was frosty. A total of 1482 progeny was produced by these 37 double-mated females, or an average of only 40.04 per female. While in *D. melanogaster* LEFEVRE and JONNSON (1962) have shown that in double-mated females the sperm from the second mating may replace that from the first, this is probably not the case in *D. euronotus*, since 775 progeny were derived from the first matings, and 707 from the second matings. It is possible that the apparent difference between the two species is associated with the normally small sperm load in *D. euronotus*, so that the relatively uncrowded sperm receptacles are able to accept and retain sperm from multiple matings without displacement.

When it is considered that under laboratory conditions the double-mated *D. euronotus* females use up approximately 99% of the sperm they carry within six days of the first mating, it seems highly probable that in nature females of this species must have to mate repeatedly if they are to maintain an adequate production of fertilized eggs.

Frequencies of multiple insemination in wild females

If a wild-inseminated female is allowed to reproduce in the laboratory and the maternal genotype compared with that of the progeny produced, it is sometimes possible to determine the sperm used, and thus the genotype of the wild male or males involved.

Multiple insemination of females in natural populations of Drosophila has apparently been reported only twice. In *D. pseudoobscura* ANDERSON (1974) found that 6 out of 15 wild females gave evidence of multiple matings on the basis of inversions found among their progeny. The author points out that the real frequency of multiple insemination must have been higher than the observed 40%, since certain types of multiple-mating would not be detected by this method. In wild females of *D. melanogaster* MILKMAN and ZEITLER (1974) used allelic differences at three enzyme loci to demonstrate multiple insemination. They avoided the complications of heterozygosis in wild females by considering only progeny of females homozygous at two of the three loci. Of 37 progenies from such females, 7 gave evidence of multiple paternity, leading to an estimate corrected for ascertainment) of 47% multiple inseminations for the population.

A comparison of the sex-linked APH genotypes of wild-inseminated *D. euronotus* females with those of 3 of their daughters indicated that among 295 wild females from five population samples, 103 or 35% of the females had mated at least twice, and 6 of these females had mated at least three times prior to capture. The real rate of multiple insemination is clearly much higher than the observed 35%, since many genotypic combinations of wild males and females will not reveal multiple insemination at all, and in other cases the three-daughter sample is insufficient to show more than one sperm type, even if several types are present.

Since the available data do not permit an accurate estimate of the relative proportions of double, triple, etc. matings, it is not possible to make an accurate estimate of the real frequency of multiple matings in the population. However, calculations based on the assumptions that all multiple-mated females were double-mated, or that all were triple-mated, led to estimates of 91% and 73% multiple-mating respectively. The true frequency probably lies somewhere between these two estimates.

As MINAMORI and FUKUI (1970) have pointed out, an obvious advantage of multiple-mating is an increased opportunity for genetic recombination, especially within small populations. *D. euronotus* gains the benefits of such increased recombination, apparently without sperm loss by displacement.

Multiple-matings without sperm displacement may also occur in the members of the *D. nasuta* subgroup of the Hawaiian Drosophila (SPEITH 1969). In these species it has been observed in the laboratory that mated females do not remate while they still carry sperm in their receptacles. Males may approach such inseminated females, and investigate them by tarsal-tapping, but then withdraw without further courting. However, once the sperm supply is exhausted, rematings occur readily.

Although sperm displacement in the laboratory in *D. melanogaster* is well established, its significance and extent in nature is unclear. In the laboratory, female receptivity to males is reduced when they carry a full sperm load, and males that mate repeatedly over a short period of time finally deliver very few sperms per mating (LEFEVRE and JONNSON 1962). If wild females were able to maintain essentially full receptacles at all times, then one might predict extensive sperm loss through displacement, even if the males involved were sexually

fatigued. In fact wild females do not mate often enough, or effectively enough to maintain full receptacles. The maximum number of sperm in inseminated laboratory females of *D. melanogaster*, determined by actual sperm count, is 650 (KAPLAN, TINDERHOLT and GUGLER 1962). Among 140 wild *D. melanogaster* females studied by STALKER (1976) the maximum effective sperm load found was 660, but the average load was only 319, and 49% of these females had an effective sperm load of less than 300, i.e. they were less than half-full. If most wild females refrain from remating until their load is seriously depleted, and/or if a significant number of matings involve sexually fatigued males that transmit little sperm, there could be relatively little sperm loss by displacement in nature.

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