References and Notes

1. M. H. F. Wilkins, A. E. Blaurock, D. M. Engelman, *Nature (London) New Biol.* **230**, 72 (1971).
2. J. B. Stamatoff, S. Krimm, N. R. Harvie, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 531 (1975).
3. E. H. Pape, K. Klott, W. Kreutz, *Biophys. J.* **19**, 141 (1977).
4. T. L. Steck, *J. Cell Biol.* **62**, 1 (1974).
5. J. T. Dodge, C. M. Mitchell, D. J. Hanahan, *Arch. Biochem. Biophys.* **100**, 119 (1963). Dodge membranes in 5.3 m*M* phosphate buffer (*p*H 7.6) were prepared from type A+ human blood within 3 days after it was drawn in citrate-phosphate-dextrose solution. Immediately after preparation, the ghosts were resealed by raising the ionic strength of the milieu to 50 m*M* with NaCl and incubating for 1 hour at 37°C. The resealed ghosts were isolated over 10 percent dextran (89,000 daltons) at $10^5 g$. These ghosts exclude large molecules from their interiors (*6*). The resealed ghosts were treated with 1 percent glutaraldehyde (final concentration) for 1 hour and washed in 50 m*M* buffer. A 5-ml portion of the membrane suspension (approximately 6 mg/ml, dry weight) was added to 5 ml of a solution (7.5 mg per 100 ml) of poly(L-lysine), centrifuged overnight at $10^5 g$ onto a flat surface, and equilibrated with a nitrogen–water vapor atmosphere over a saturated solution of KCl (86 percent relative humidity at 22°C). Diffraction patterns were recorded on film and with a position-sensitive detector. Resealed and partially dehydrated membranes with and without poly(L-lysine) (final concentration, 25 mg per 100 ml) were fixed with glutaraldehyde and OsO_4 before embedding in Epon-Araldite. Electron micrographs were taken of thin sections after staining with uranyl acetate and lead citrate.
6. R. M. Johnson, *J. Membr. Biol.* **22**, 231 (1975).
7. J. B. Finean, *Prog. Biophys. Mol. Biol.* **16**, 143 (1966).
8. L. McCaughan and S. Krimm, *Biophys. J.* **25**, 93a (1979).
9. L. McCaughan, thesis, University of Michigan (1979). Earlier membrane specimens that were similarly treated sometimes showed a diffraction peak near 1/55 Å^{-1}. The conditions determining the appearance of this peak are not yet clear.
10. A. Katchalsky, D. Danon, A. Nevo, A. DeVries, *Biochim. Biophys. Acta* **33**, 120 (1959).
11. This is supported by two lines of evidence. First, resealed ghosts remain suspended above a pad of 10 percent dextran of similar molecular weight (89,000) during ultracentrifugation; "leaky" ghosts are pelleted. Second, if the 37°C incubation is omitted from the resealing procedure, the ghosts remain leaky (*6*). Addition of poly(L-lysine) to these leaky ghosts before partial dehydration produces a set of membranes organized on a 190-Å lattice whose spacing cannot be varied by moderate changes in the relative humidity used for partial dehydration. [It should be noted that the cytoplasmic protein spectrin provides about the same amount of negative charge for interaction with poly(L-lysine) as do the sialic acid residues at the extracellular surface (*12*).] On the other hand, resealed ghosts treated with poly(L-lysine) produced Bragg spacings that vary with hydration from ~180 to 275 Å.
12. A. Elgsaeter, D. Shotton, D. Branton, *Biochim. Biophys. Acta* **426**, 101 (1976).
13. Film recordings of x-ray diffraction patterns and ω scans of neutron diffraction patterns with a two-dimensional detector show that the mosaic disorder present in the specimens is accurately represented by a cylindrically symmetric Gaussian intensity distribution with a characteristic width ($\Delta\omega$ = 16°, full width at half-maximum) that is independent of scattering angle (2θ). A Lorentz correction of S^2, where S is a reciprocal space coordinate, was therefore made to the diffraction data after correcting for beam and detector geometries.
14. S. Schwartz, J. E. Cain, E. A. Dratz, J. K. Blasie, *Biophys. J.* **15**, 1201 (1975). Because both substitutional and lattice disorder were substantial, zeros (points of possible phase inversion) of the transform could not be assigned confidently by inspection. Instead, the data were first fit to a sampling function with simple lattice disorder. All possible sets of phases were applied to the "reflections" to obtain a set of trial profiles. An iterative method similar to that described by Schwartz *et al.* was used to determine the scattering due to substitutional disorder, to refine the sampling function, and to locate the zeros of the transform. The continuous transform (not the generalized Patterson function Q_0) was used as the criterion for correct phasing. One point of phase inversion was found at reciprocal space coordinate S = 1/48 Å^{-1} for the x-ray data. Data were reliably recorded to S = 1/24 Å^{-1}. The neutron diffraction data produced no phase inversion below 1/40 Å^{-1} for the data for either 100 percent or 75 percent D_2O.
15. R. P. Rand and V. Luzzati, *Biophys. J.* **8**, 125 (1968).
16. J. B. Finean, R. Freeman, R. Coleman, *Nature (London)* **257**, 718 (1975).
17. V. T. Marchesi and E. Steers, Jr., *Science* **159**, 203 (1968).
18. B. P. Schoenborn, J. Alberi, A. M. Saxena, J. Fischer, *J. Appl. Crystallogr.* **11**, 455 (1978).
19. L. McCaughan and S. Krimm, in preparation.

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Esterase 6 and Reproduction in *Drosophila melanogaster*

Abstract. *A nonspecific carboxylesterase (esterase 6) of* Drosophila melanogaster *shows greater activity in adult males than in females and is highly concentrated in the anterior ejaculatory duct of the reproductive tract of the male. Esterase 6 is depleted in males by copulation and is transferred to females early during copulation as a component of the seminal fluid. That esterase 6 may be involved in a system controlling the timing of remating is suggested by differences in the activity of this enzyme in a strain of* Drosophila *selected for a decrease in time to remating and by differences in the timing of remating in females initially inseminated by males lacking or having active esterase 6.*

Esterases are among the most genetically variable enzymes known in both plants and animals (*1*). Carboxylesterases (E.C. 3.1.1) have been extensively studied in *Drosophila* and are highly polymorphic (*2*). The first biochemical polymorphism discovered in *Drosophila* was a carboxylesterase, esterase 6 (EST 6), found in *D. melanogaster* (*3*), and genetic variation at the autosomal *esterase 6 (Est 6)* locus has been found in every natural population studied (*1*). With few exceptions (*4*), however, the functions of nonspecific esterases and their in vivo substrates are unknown. Studies (*5*, *6*) on the developmental pattern and tissue distribution of EST 6 have suggested that this enzyme might have a function in some aspect of reproduction. We present results here which show that EST 6 is a component of the seminal fluid of *D. melanogaster*, is active in the reproductive tract of females, and may act to influence the timing of female remating.

Natural populations of *D. melanogaster* are invariably polymorphic for two electrophoretic variants of EST 6 termed EST 6^F and EST 6^S. Two strains of flies derived from a natural population in Bloomington, Indiana, were individually made homozygous for the third chromosome, which carries the structural locus for EST 6. This procedure ensures that only one form of EST 6 is present in flies from either of these two strains. Flies heterozygous for the *Est* 6^F and *Est* 6^S alleles were obtained from a balanced lethal strain carrying the dominant morphological markers, *Stubble* and *Serrate* (*7*). A null stock (*Est* 6^o) lacking esterase activity and producing no detectable EST 6 protein has been described (*6*).

Several investigators have shown that the specific activity of EST 6 may be fivefold greater in 3- to 5-day-old virgin males than in virgin females of the same age (*5*, *6*). In males, the enzyme is highly concentrated in the anterior ejaculatory duct, a reproductive tract, secretory organ connecting the accessory glands and testes to the ejaculatory bulb and penis (*5*, *6*). The possibility that EST 6 is involved in reproduction was tested by determining whether the enzyme was depleted in males by copulation and transferred to females. Virgin males were collected from a stock homozygous for the *Est* 6^S allele, and virgin females were collected from the *Est* 6^o stock. All flies were aged at 25°C for 4 days. The males were divided into six groups of ten flies each. Two groups of males (treatment A) and two groups of females (treatment D) were immediately frozen at −50°C. Two groups of males (treatment B) were indi-

Table 1. Esterase 6 activity (± the standard error of the mean) in virgin and mated *Drosophila melanogaster*. The number of flies in each group was ten.

Treatment	Groups (No.)	Specific activity of EST 6*
A. Virgin *Est* $6^{S/S}$ ♂♂	2	3.289 ± 0.317
B. Once mated *Est* $6^{S/S}$ ♂♂	2	1.046 ± 0.125
C. Twice mated *Est* $6^{S/S}$ ♂♂	2	0.778 ± 0.173
D. Virgin *Est* $6^{o/o}$ ♀♀	2	0.042 ± 0.004
E. *Est* $6^{o/o}$ ♀♀ mated to *Est* $6^{S/S}$ ♂♂	3	0.185 ± 0.035

*Micromoles of β-naphthol per milligram of protein per 30 minutes at 27°C.

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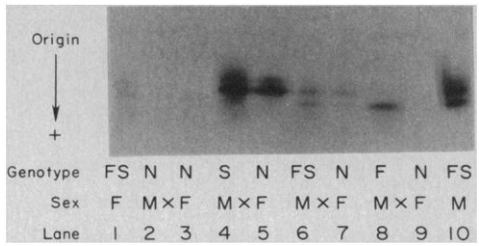

Fig. 1. Acrylamide-gel electrophoresis of *Est* $6^{o/o}$ (N) females mated to *Est* $6^{F/F}$ (F, lane 8), *Est* $6^{S/S}$ (S, lane 4), *Est* $6^{F/S}$ (FS, lane 6), and *Est* 6^{o} (N, lane 2) males. Channels 1 and 10 contain a virgin *Est* $6^{F/S}$ female and male, respectively. Flies were individually homogenized (*18*) in 15 μl of distilled water and the homogenate was diluted to 50 μl with distilled water. The individual fly homogenates were centrifuged at 15,600*g* for 5 minutes. Immediately after centrifugation, the supernatants were placed in the wells of a 6 percent acrylamide-slab gel 0.8 mm thick and subjected to electrophoresis in the buffer system of Davis (*19*). After electrophoresis, the gel was stained for esterase activity with both α- and β-naphthyl acetates as substrates (*18*).

vidually placed with virgin *Est* 6^{o} females until copulation was observed. After mating, the sexes were separated and immediately frozen. The females form treatment group E. In the treatment C groups, individual males placed with two virgin *Est* 6^{o} females were permitted to mate once, individually transferred to another vial containing females, and allowed to mate again before being frozen. All groups of flies were later analyzed for total protein and EST 6 activity (*6*). A one-way analysis of variance of the EST 6 specific activity of the male groups (Table 1) shows these treatment means are significantly heterogeneous [$F_{(2,3)} = 39.1$, $P < .01$]. A Student-Newman-Keuls test (*8*) of means reveals that virgin males have significantly higher EST 6 activity than either once- or twice-mated males, and that once- and twice-mated males do not differ significantly. These results demonstrate that EST 6 activity is depleted in males as a result of copulation, and that little, if any, additional loss of activity occurs if a male remates immediately. A comparison of treatment groups D and E reveals that EST 6 activity in recently mated females is significantly above that of the controls ($t = 3.2$, $P < .05$).

Sufficient EST 6 activity is present in mated *Est* 6^{o} females so that the enzyme can be detected after electrophoresis of homogenates of individual females. Four-day-old virgin males of four *Est* 6 genotypes ($6^{S/S}$, $6^{S/F}$, $6^{F/F}$, $6^{o/o}$) were mated to individual *Est* 6^{o} females. After the matings were completed, each individual was homogenized, the homogenates were centrifuged, and the supernatants were placed in the wells of a thin-layer, acrylamide-gel slab (Fig. 1). Lanes 2 and 3 of this gel contained the homogenates of an *Est* 6^{o} female and her *Est* 6^{o} mate. No EST 6 activity is present. However, in lanes 5, 7, and 9 containing homogenates of *Est* 6^{o} females mated to males homozygous and heterozygous for the *Est* 6^{F} and *Est* 6^{S} alleles, the female sample shows EST 6 activity of the same type as the male with which she was mated. (The *Est* 6^{F} strain utilized in this experiment has reduced activity and the *Est* 6^{o} female band is difficult to see in a photograph.) Thus, the depletion of EST 6 in males on mating is due to its transfer to females in the seminal fluid. Additional experiments with the gel assay for EST 6 show that the enzyme can be detected in *Est* 6^{o} females after only 1 minute of copulation with virgin *Est* $6^{S/S}$ males. This result is interesting since sperm do not begin to be transferred until 4 to 8 minutes after copulation is initiated (*9*). Apparently the first few minutes of a normal 20-minute copulation are devoted to the transfer of substances other than sperm.

The role of EST 6 in the reproduction of flies is being studied. Several lines of evidence led us to test the hypothesis that EST 6 might be involved in a system that allows the male to influence the timing of female remating. Females of *D. melanogaster* will remate before sperm from a previous mating is exhausted (*10*).

Table 2. Cumulative numbers and frequencies (in parentheses) of females remating per day after initially mating with an *Est* $6^{o/o}$ or *Est* $6^{S/S}$ male. Females homozygous for the recessive, sex-linked, allele, *forked*, were used as a control for nonvirginity. Females were given the opportunity to remate with the males of the *forked* strain.

Time after first mating (days)	Cumulative frequencies* of females first mated to	
	Est $6^{o/o}$ male	*Est* $6^{S/S}$ male
1	7 (0.233)	6 (0.207)
2	8 (0.267)	11 (0.379)
3	13 (0.433)	22 (0.759)
4	20 (0.667)	26 (0.897)
5	23 (0.767)	26 (0.897)
6	26 (0.867)	28 (0.966)
7	28 (0.933)	29 (1.00)
8	28 (0.933)	29 (1.00)
9	28 (0.933)	29 (1.00)
N	30	29

*Kolmogorov-Smirnov (*8*) one-sided test of the hypothesis that the two cumulative distributions were drawn from the same underlying distribution yielded $D = 0.326$; $\chi^2_2 = 6.27$; $.025 > P > .01$.

Since much of the stored sperm remaining at the time of remating is displaced by sperm from a later male (*10*), a male-controlled mechanism delaying female remating as long as possible would be adaptive (*11*). Manning (*12*) showed that inhibition of sexual receptivity in mated *Drosophila* females had two components: (i) an effect associated with the presence of sperm in the females' storage organs and (ii) an effect due to copulation itself which he hypothesized might be caused by some of the constituents of the seminal fluid.

In an independent investigation, Pyle and Gromko (*13*) selected a strain of *D. melanogaster* for decreased time to remating. The selected line of flies (MMS) remated approximately 2 days after its initial mating while an equivalently inbred but unselected control line (CON) originating from the same base population remated after an average of more than 5 days. Both lines are homozygous for *Est* 6^{S}. The EST 6 activity in these lines was examined in virgin males aged from 0 (late pupal stage) to 20.5 days (*6*). At every sample point after the animals were 1 day of age, the MMS strain has significantly more EST 6 activity than the control strain. This result is consistent with the hypothesis that EST 6 is part of a system that influences the timing of female remating.

This hypothesis was tested further by comparing the time to remating in females initially inseminated by *Est* $6^{o/o}$ or *Est* $6^{S/S}$ males. Virgin females were inseminated with males of either EST 6 type and tested for remating for 2 hours on each day thereafter with a third type of male. The results, expressed as a cumulative remating distribution (Table 2), show that females initially inseminated by *Est* $6^{S/S}$ males remate sooner than females initially inseminated by *Est* $6^{o/o}$ males. These two pieces of information suggest that increased EST 6 activity results in a reduction in time to remating.

The mechanism by which EST 6 might influence the timing of remating is unclear at present. The discovery that *D. melanogaster* males produce a sex-specific lipid (male lipid) localized in the ejaculatory bulb and transferred to females during copulation (*14*) suggested a possible candidate for the substrate of EST 6 or the product of an EST 6 catalyzed reaction. The male lipid is an acetate ester of the fatty acid, *cis*-11-octadecenoic acid, and has also been isolated from reproductive organs of male Danaid butterflies where it is required for successful copulation (*15*). Several carboxylesterases, and in particular EST 6, act as lipolytic enzymes under the appropri-

ate conditions (*16*). To test the possibility that EST 6 is involved in the production of the male lipid, extracts of males from several strains of *D. melanogaster* homozygous for the *Est* 6^S and *Est* 6^o alleles were subjected to thin-layer chromatography (*14*). The male lipid was clearly present in strains carrying both the null and active alleles, an indication that the enzyme is not involved in the production of this substance.

Gilbert *et al.* (*17*) have shown that the number of sperm stored and its utilization by females differs according to the EST 6 type of the inseminating male. Although the total number of progeny produced by females inseminated by *Est* $6^{S/S}$ or *Est* $6^{o/o}$ males is not significantly different, more sperm are stored in *Est* $6^{0/0}$ inseminated females and sperm is initially used at a greater rate by *Est* $6^{S/S}$ inseminated females. These results suggest that EST 6 may influence remating by affecting both the storage and utilization of sperm. The possibility remains that EST 6 may interact with the male lipid in the female's reproductive tract to achieve these effects.

The widespread occurrence of nonspecific esterase polymorphisms in many plant and animal populations has made them valuable tools for systematic, genetic, population genetic, and medical studies. However, an almost complete absence of information about their functions in vivo limits their utility. Our results show that *D. melanogaster* is viable without EST 6, but that the enzyme may be involved in physiological control of reproduction in *Drosophila*. Although the relation between genetic variation at the *Est 6* locus and possible differences in individual fitness are unknown (*17*), our observations provide a system in which to begin an experimental study of this relation.

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References and Notes

1. J. R. Powell, *Evol. Biol.* **8**, 79 (1975); E. Nevo, *Theor. Pop. Biol.* **13**, 121 (1978).
2. T. R. F. Wright and R. J. MacIntyre, *Genetics* **48**, 1717 (1963); F. M. Johnson, C. G. Kanapi, R. H. Richardson, M. R. Wheeler, W. S. Stone, *Univ. Tex. Publ. Genet. No. 6615*, 517 (1966); J. Coyne, A. A. Felton, R. C. Lewontin, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5090 (1978).
3. T. R. F. Wright, *Genetics* **48**, 787 (1963).
4. D. Whitmore Jr., E. Whitmore, L. J. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1592 (1972); E. Berger and R. Canter, *Dev. Biol.* **33**, 48 (1973); R. G. H. Downer, M. Wiegand, S. M. Smith, *Experientia* **31**, 1239 (1975).
5. A. D. Aronshtam and B. A. Kuzin, *Zh. Obsch. Biol.* **35**, 926 (1974).
6. K. Sheehan, R. C. Richmond, B. J. Cochrane, *Insect Biochem.* **9**, 443 (1979).
7. D. L. Lindsley and E. H. Grell, *Genetic Variations of Drosophila melanogaster* (Carnegie Inst. of Washington Publ. No. 627 (1968).
8. R. R. Sokal and F. J. Rohlf, *Biometry* (Freeman, San Francisco, 1969), pp. 239-246.
9. A. Garcia-Bellido, *Z. Naturforsch. Teil B* **19**, 491 (1964); A. Bairati, *Monit. Zool. Ital.* **2**, 105 (1968).
10. G. Lefevre and U. B. Jonsson, *Genetics* **47**, 1719 (1962); M. H. Gromko and D. W. Pyle, *Evolution* **32**, 588 (1978).
11. G. A. Parker, *Biol. Rev.* **45**, 525 (1970).
12. A. Manning, *Nature (London)* **194**, 252 (1962); *Anim. Behav.* **15**, 239 (1967).
13. D. W. Pyle and M. H. Gromko, *Am. Nat.*, in press.
14. F. M. Butterworth, *Science* **163**, 1356 (1969); G. Brieger and F. M. Butterworth, *ibid.* **167**, 1262 (1970).
15. T. E. Pliske and T. Eisner, *ibid.* **164**, 1170 (1969); Y. Tamaki, in *Chemical Control of Insect Behavior*, H. H. Shorey and J. J. McKelvey, Jr., Eds. (Wiley, New York, 1977), p. 253.
16. L. I. Gilbert, H. Chino, K. A. Domroese, *J. Insect Physiol.* **11**, 1057 (1965); M. P. Castillon, R. E. Catalan, S. Vega, A. M. Municio, *Comp. Biochem. Physiol.* **44B**, 639 (1973); N. D. Danford and J. A. Beardmore, *Biochem. Genet.* **17**, 1 (1979).
17. D. G. Gilbert, R. C. Richmond, K. S. Sheehan, in preparation.
18. R. C. Richmond, *Genetics* **70**, 87 (1972).
19. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
20. We thank C. Nelson, P. Randolph, A. Richmond, and P. Vitousek for reading the manuscript. Supported by NSF grant DEB 77-14994 and by PHS grant S07 RR 7031 to R.C.R.

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Food Dyes Impair Performance of Hyperactive Children on a Laboratory Learning Test

Abstract. *Forty children were given a diet free of artificial food dyes and other additives for 5 days. Twenty of the children had been classified as hyperactive by scores on the Conners Rating Scale and were reported to have favorable responses to stimulant medication. A diagnosis of hyperactivity had been rejected in the other 20 children. Oral challenges with large doses (100 or 150 milligrams) of a blend of FD &C approved food dyes or placebo were administered on days 4 and 5 of the experiment. The performance of the hyperactive children on paired-associate learning tests on the day they received the dye blend was impaired relative to their performance after they received the placebo, but the performance of the nonhyperactive group was not affected by the challenge with the food dye blend.*

Feingold (*1*) hypothesized that artificial food dyes are pharmacologically active substances that induce or aggravate symptoms of hyperactivity in children. Even though a controlled double-blind study of 46 hyperactive children (*2*) and a series of open trials involving 142 hyperactive children (*3*) have confirmed that about 50 percent of those tried on the Feingold diet showed a decrease in symptoms of hyperactivity, the mechanism for and the magnitude of this effect remains in question. Some studies (*4*) have failed to document that reintroduction of (or challenge with) 1 to 26 mg of artificial food dyes (*5*) elicits symptoms of hyperactivity. In other studies, the behavioral effects of the diet (*2, 6*) or the responses to challenges with food dyes (*7*) have been considerably less in magnitude than in reports based on clinical observations (*1, 8*). For these reasons, the benefits associated with the Feingold diet have been attributed to a placebo or Hawthorne effect (*9*).

The lack of response to challenges in previous studies may have been due to an insufficient dose of dye. We have used higher challenge doses—up to 150 mg, which the Food and Drug Administration (*10*) estimated to be at the 90th percentile for daily consumption of artificial food dyes by children from 5 to 12 years of age. With these larger doses we have documented a dye-induced impairment of performance on a laboratory learning test.

We tested 40 children (36 boys and 4 girls; average age, 10 years) referred to the Child Development Clinic of the Hospital for Sick Children with behavioral symptoms suggesting hyperactivity (for example, short attention span, aggressiveness, overactivity, impulsivity, distractibility). To form these groups, we selected 20 children who had shown a favorable response to stimulant medication (*11*) and 20 children in whom an adverse response had been documented. The group of favorable responders had an average score of 16.2 in the Conners Rating Scale (*12*), and the group of adverse responders had an average score of 12.3, which is under the criterion of 15 established for the diagnosis of hyperactivity. Thus, one group was composed of children on whom the diagnosis of hyperactivity had been confirmed, and the other group was composed of children for whom a diagnosis of hyperactivity had been rejected.

The children were admitted to the Clinical Investigations Unit (CIU) of the hospital in pairs matched for age and sex

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