STUDIES OF ESTERASE 6 IN *DROSOPHILA MELANOGASTER*. VI. EJACULATE COMPETITIVE ABILITIES OF MALES HAVING NULL OR ACTIVE ALLELES

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ABSTRACT

Recent studies of the function of the polymorphic seminal fluid enzyme, esterase 6, of *Drosophila melanogaster* suggested that it may act in the process of sperm displacement (GILBERT, RICHMOND and SHEEHAN, 1981a). This report examines the competitive ability of ejaculates from males homozygous for null or active alleles of esterase 6 under three experimental conditions that model aspects of sexual selection affecting males. The results demonstrate no significant difference in ejaculate competition between esterase 6 null or active male types, but marker males used for paternity identification had poorly competitive ejaculates. The proportion of second-male progeny, P_2, used as an index of competition is primarily influenced by second-male genotype and uninfluenced by female genotype. P_2 can change with time from remating and be unaffected by different intensities of competition, which suggests a complex ejaculate competition mechanism.

GENETIC variation at the esterase 6 (*Est-6*) locus is common in natural and laboratory populations of *Drosophila melanogaster* and appears to constitute a stable polymorphism for the two major electrophoretic variants (GIRARD, PALABOST and PETIT 1977; BAND 1975; SMITH, LANGLEY and JOHNSON 1978). The product of this locus, esterase 6 (EST-6), has recently been identified as a male reproductive enzyme, based on its localization in the adult male anterior ejaculatory duct (SHEEHAN, RICHMOND and COCHRANE 1979), its transmission to females in the ejaculate and its effect on the timing of remating in females (RICHMOND *et al.* 1980).

Our observations (GILBERT, RICHMOND and SHEEHAN 1981a; GILBERT, submitted) that EST-6 is transferred in the ejaculate prior to sperm transfer and increases sperm release from female storage organs suggested that EST-6 might function in ejaculate competition as a sperm-displacing enzyme. Displacement of sperm from the female sperm-storage organs is one possible adaptation by which males can compete to fertilize ova and has been demonstrated in the damselfly (WAAGE 1979). Direct competition between two ejaculates may rely on displacement, positional precedence of sperm in the storage organs, sperm incapacitation, differences in sperm motility and fertilizing ability or other adaptations, which may also result in the nonrandom use of sperm within single ejaculates (PARKER

1970; BOORMAN and PARKER 1976; CHILDRESS and HARTL 1972; FOWLER 1973). Ejaculate competition has been demonstrated in doubly inseminated *D. melanogaster* females through a loss in the potential number of offspring sired by the females' first mates (GROMKO and PYLE 1978; BOORMAN and PARKER 1976), and genetic variation for this trait is found associated with morphologically marked sperm used for paternity identification (PROUT and BUNDGAARD 1977; LEFEVRE and JONSSON 1962; LOBASHOV 1939).

In this report, we compare ejaculate competitive abilities of males that are homozygous for the *Est-6*^{o} (null) and *Est-6*^{s} (slow, active) alleles when tested with females previously inseminated with sperm carrying morphological marker genes. Three mating designs vary the degree of competition, and two marker stocks (forked and carnation) vary the tester ejaculates to rule out an isolated effect. Ejaculate competition is measured in terms of productivity (progeny per female) following remating and the proportion of progeny sired by the second male, P_2 (BOORMAN and PARKER 1976). A significantly lower P_2 for *Est-6*^{o} compared to *Est-6*^{s} males would demonstrate EST-6 role in ejaculate competition, presumably through its effect on stored sperm as a displacement enzyme (GILBERT, RICHMOND and SHEEHAN 1981a).

MATERIALS AND METHODS

Drosophila stocks and husbandry: Four inbred *Drosophila melanogaster* stocks were used in the experiments.

(1) *ss*—homozygous for the *Est-6*^{s} allele.

(2) *oo*—homozygous for the *Est-6*^{o} allele, a null variant.

(3) *f ss*—homozygous for the sex-linked allele *f* (forked bristles) and the *Est-6*^{s} allele.

(4) *car oo*—homozygous for the sex-linked allele *car* (carnation eye color) and *Est-6*^{o}.

Stocks 1 and 2 have similar genetic backgrounds and were derived as described by SHEEHAN, RICHMOND and COCHRANE (1979). Stocks 3 and 4 were used to provide morphologically marked offspring for paternity determinations. Stock populations and experimental flies were housed at 25°, 60% relative humidity and under a 12:12 hr light/dark cycle on an agar, cornmeal, molasses and yeast medium.

Experiment 1: This experiment was designed to test the effect of the *Est-6* genotype of a female's second mate on the degree of sperm displacement and the timing of remating. An outline of the experimental design is provided in Table 1. Seventy virgin *f ss* males were individually mated to virgin *f ss* females, and the females were subsequently divided into 2 groups, A and B. Each female was transferred to fresh-yeasted medium daily and provided with a virgin *ss* male (group A) or a virgin *oo* male (group B) for 2 hr daily until remating occurred. Rematings were scored visually, and each female was transfered to a fresh vial daily for 7 days following remating. All progeny from each post-remating vial were counted 18 days after eggs were first laid. Progeny were scored as male (all forked), forked female (first male paternity), and wild-type female (second male paternity). This mating design may be considered as a simple model of mating dynamics in which female acceptance controls the degree of remating in nature.

Experiment 2: This experiment is designed to measure male reproductive success in which males may compete directly with other males, as well as through ejaculates, and in which they may court and copulate with females in an unrestricted manner. Adult flies in this experiment were random samples of the stock populations and presumably nonvirgin. In each of 5 groups with 8 replicate crosses per group, 10 *f ss* females were placed in a half-pint bottle with yeasted media for 7 days. The female offspring were counted for 9 days after flies first eclosed and

TABLE 1

Summary of mating designs for experiments 1, 2 and 3

	Genotype of				
	Female	First male	Second male	code	No. ♀♀
Experiment 1	*f ss*	*f ss*	*ss*	1A	32
	f ss	*f ss*	*oo*	1B	34
Experiment 2	*f ss*	*f ss*	none	2A	80
	f ss	*f ss*	*ss*	2B	80
	f ss	*f ss*	*oo*	2C	80
	f ss	*f ss*	*ss* and/or *f ss*	2D	80
	f ss	*f ss*	*oo* and/or *f ss*	2E	80
Experiment 3	*f ss*	*ss*	*f ss*	3A	3
	f ss	*oo*	*f ss*	3B	8
	f ss	*car oo*	*f ss*	3C	4
	f ss	*f ss*	*ss*	3D	7
	f ss	*f ss*	*oo*	3E	7
	f ss	*f ss*	*car oo*	3F	2
	car oo	*ss*	*car oo*	3G	8
	car oo	*oo*	*car oo*	3H	4
	car oo	*f ss*	*car oo*	3I	4
	car oo	*car oo*	*ss*	3J	8
	car oo	*car oo*	*oo*	3K	8
	car oo	*car oo*	*f ss*	3L	5

scored as forked or wild-type. Group A (Table 1) was a control for fertility of the nonvirgin females. Groups B and C had 10 *ss* males or 10 *oo* males housed with the 10 females. Groups D and E had 5 *ss* males plus 5 *f ss* males or 5 *oo* males plus 5 *f ss* males. The designation of forked and wild-type offspring as first and second male progeny is approximate for groups D and E where forked males mate before, after or instead of wild-type males.

Experiment 3: In this experiment, individual virgin females of the *f ss* and *car oo* stocks were mated to a first and second male of the *ss*, *oo*, *f ss* and *car oo* stocks in the 12 combinations for which first and second male paternity can be distinguished by the morphological markers (Table 1). Each female was housed with the first male for 24 hr, then transferred to a fresh vial with the second male for the next 24 hr and subsequently transferred alone for 8 days. Since *D. melanogaster* females rarely mate more than once in 24 hr when housed continually with males (BUNGAARD and CHRISTENSEN 1972; MCSHEEHY 1963), even when mated to sterile males (MANNING 1967), this design effectively tests the maximum degree of competition between two ejaculates.

This experiment also tests the ejaculate competitive ability of the 4 male types to allow an assessment of the possible significance of the morphological marker in ejaculate competition. 5 to 10 replicates in each group were started but, since matings were not observed, only females producing offspring from both males were retained for analysis. All progeny were counted by 18 days after egg laying began and scored as male, forked, carnation or wild-type females. Because progeny from the second transfer day, when the second male was present, may be a combination of pre- and post-remating offspring, only progeny from days 3 to 10 are included in the analysis.

Analysis: The effects of ejaculate competition on male fitness are analyzed in terms of two parameters: total offspring following remating and P_2, the proportion of second male offspring.

TABLE 2

Fitness component statistics for experiment 1

Group	Days to remating		Post-remating offspring		P_2†	
	ss	*oo*	*ss*	*oo*	*ss*	*oo*
Mean	4.59	4.65	183.0	185.8	0.967 (79.5°)	0.941 (75.9°)
Standard error of mean	0.249	0.235	12.40	13.92	1.48°	2.09°
t statistic*	0.156		0.149		1.409	

† P_2, the proportion of second-male offspring, is given as the back-transformed proportion (and angular mean in parentheses) and standard error of the angular mean.
* Values are not statistically significant.

For experiment 1, time to remating was also determined as a possible component of male reproductive fitness other than ejaculate competition. P_2 values for individual crosses have been transformed to their angular values for analysis to reduce the asymmetry commonly associated with distributions of proportions (BLISS 1967). P_2 results are reported in terms of the angular mean, the standard error of the mean, and the back-transformed proportional mean. The analyses of variances reported were performed as suggested by BLISS (1967, 1970). Daily changes in P_2 were compared among groups in experiments 1 and 3 to gather indirect information on the mechanism of ejaculate competition.

RESULTS

Total productivity and P_2

Experiment 1: There were no significant differences in number of days to remating, number of post-remating offspring or proportion of second male offspring, P_2, for *f ss* females whose second mate was an *ss* or *oo* male (Table 2). The *f ss* females remated with *ss* or *oo* males in an average of 4.6 days, producing an average of 184 offspring during the seven days following remating. Both male types sired an average of 95% of the post-remating offspring. The small difference in P_2 is in the direction predicted by GILBERT, RICHMOND and SHEEHAN (1981a), but clearly not of a magnitude that would implicate EST-6 as a major factor in ejaculate competition.

Experiment 2: Analyses of the number of post-remating female offspring produced by each group are given in Table 3. The means recorded in Table 3A suggest that the presence of males with females (groups B-E) may result in a re-

TABLE 3A

Mean number of post-remating female offspring and their standard errors (SEM) for experiment 2

Group	No. female offspring ± SEM
A. No 2nd males	203.0 ± 11.2
B. 10 *ss* males	181.0 ± 14.6
C. 10 *oo* males	182.6 ± 10.7
D. 5 *ss* and 5 *f ss* males	161.6 ± 13.6
E. 5 *oo* and 5 *f ss* males	174.6 ± 12.2

TABLE 3B

Analysis of the variance in female offspring number for experiment 2

Source	df	MS	*F*
2nd male present or absent	1	5028.81	3.99*
Between 2nd male groups	3	728.45	0.58
Within groups	35	1261.53	

Level of significance: $*p = 0.054$.

duction in the number of progeny produced. An analysis of the variance in post-remating offspring number (Table 3B) reveals that there are no significant productivity differences due to the second male group, but the reduction in progeny with the presence of males is significant at the 0.05 level, suggesting that courtship interactions inhibit egg laying. Table 4A reports means P_2 values for groups B-E. The average P_2 values of 0.95 for groups B and C are nearly identical to those found in experiment 1 (Table 2). The P_2 values for groups B and C are not significantly different; but, in contrast to the results of experiment 1, *oo* second males produced slightly more post-remating progeny than did *ss* males. This result shows that the absence of a significant difference in ejaculate competitive ability found in experiment 1 was not solely a result of the mating design employed.

The P_2 values for the mixed-male groups D and E (Table 4A) are well below those for the single-male groups B and C, indicating a significant effect due to male competition in these groups (Table 4B). When P_2 values are partitioned according to the presence of competitor males (groups B, C *vs.* D, E), a highly significant difference is detected. This difference might be a result of differences in courtship abilities or in the ejaculate competition abilities of *ss* and *oo vs. f ss* males. The significant esterase 6 effect (Table 4B) results from the lower P_2 for group D compared to group E and is likely due to earlier remating by *ss* inseminated females compared to *oo* inseminated females (RICHMOND *et al.* 1980; GILBERT, RICHMOND, SHEEHAN 1981b). This would lead to a greater chance of remating with *f ss* males and to a lower P_2.

The P_2 values for groups D and E are significantly greater than a maximum of 0.50 expected for equally competitive forked (*f ss*) and wild-type males (*oo* or

TABLE 4A

Mean P_2 and standard errors (SEM) for experiment 2*

Group	Mean	Angular mean ± SEM
B. 10 *ss* males	0.941	75.9 ± 1.15
C. 10 *oo* males	0.961	78.7 ± 1.49
D. 5 *ss* and 5 *f ss* males	0.733	58.9 ± 2.69
E. 5 *oo* and 5 *f ss males*	0.848	67.0 ± 2.81

* Proportion of post-remating female progeny sired by the 2nd male. In the case of groups D and E, the *ss* and *oo* males are arbitrarily treated as the second-male type.

TABLE 4B

Analysis of the variance in P_2 *for experiment 2*

Source	df	MS	*F*
2nd male groups	3	648.01	17.38‡
Esterase 6	1	237.24	6.36*†
Male competition	1	1648.38	44.20‡
Interaction	1	58.40	1.57
Within groups	28	37.29	

Levels of significance: $*p < 0.05$; $‡p < 0.001$.
† A Student-Newman-Keuls *a posteriori* test shows that groups B and C do not differ at the 0.05 level, while groups D and E differ at the 0.01 level of significance.

ss). Testing the observed angular P_2 against the hypothesis that P_2 for wild-type males is $\leq 45°$, the angular transform of 0.50, yields *t* values of 5.17 and 7.83 for groups D and E, respectively ($p < 0.001$ with 7 df for the 1-tail hypothesis). This fact, and our prior observations of the reverse ejaculate competition experiment (GILBERT, RICHMOND and SHEEHAN 1981b) with wild-type males as first and *f ss* males as second mates, suggested that forked male ejaculates are poorly competitive with wild-type male ejaculates. A third experiment was conducted to ensure that the results of experiments 1 and 2 were not due to a unique deficit of the *f ss* males.

Experiment 3. The total number of progeny and P_2 values attributable to the two females and four male genotypes (as first or second mate), independent of the genotype of the other mate, is given in Table 5. In the female class, the two genotypes show a marked difference in productivity but little difference in P_2 values. This result suggests that female genotype may not affect ejaculate competition. The largest range in mean productivity is in the first male class. The largest range in P_2 values is found among genotypes in the second male class, with *Est-6*$^{s/s}$ and *Est-6*$^{o/o}$ having nearly identical values and both sex-linked marker groups having lower P_2 values.

TABLE 5

Means and standard errors of means for total offspring number (a) and P_2 *(b) in experiment 3 according to genotype of female, first male or second male*

Genotype	Female	First male	Second male
(a) Total offspring			
ss		142.4 ± 11.74	160.4 ± 17.04
oo		121.7 ± 21.01	169.0 ± 15.61
f ss	185.9 ± 11.00	181.6 ± 14.87	175.9 ± 13.04
car oo	134.2 ± 9.90	170.1 ± 12.22	128.0 ± 15.58
(b) P_2*			
ss		0.730 (58.7 ± 7.04)	0.952 (77.3 ± 4.46)
oo		0.554 (48.1 ± 6.21)	0.957 (78.0 ± 2.26)
f ss	0.790 (62.7 ± 4.41)	0.818 (64.8 ± 5.60)	0.676 (55.3 ± 5.21)
car oo	0.811 (64.2 ± 3.71)	0.911 (72.6 ± 3.75)	0.585 (49.9 ± 5.57)

* P_2 is given as the back-transformed proportion (and angular mean ± SEM in parentheses).

An analysis of the genotype effects for the different parental classes is reported in Table 6. Due to the incomplete factorial design of this experiment, interaction of effects among the three parental classes (female, male as first and second mate) cannot be fully analyzed. However, the groups C, F, I and L form a balanced comparison for two female types and two second plus first male types. An analysis of variance of these groups shows that the female genotype effect on offspring and the male effect on P_2 are significant, but male effect on offspring, female effect on P_2, and the interaction of the female and male effects are all insignificant components of the variation.

An analysis of the whole data set (Table 6) shows that total offspring production following remating is affected by female genotype, the *X* marker carried by the first male and by the second male. The second-male effect is due primarily to an interaction between EST-6 type and the X-linked marker. Significant effects on P_2 are associated with both male types and are due primarily to the marker carried by the male. Other than a slight interactive effect, EST-6 type is not associated with ejaculate competitive ability in this experiment.

Daily change in P_2

The changes in P_2 following remating are illustrated for experiments 1 and 3 in Figure 1. Both *Est-6*$^{s/s}$ and *Est-6*$^{o/o}$ males produce a consistently high proportion of their own offspring during the initial four days after remating. The daily mean values are not significantly different between *ss* and *oo* groups for either experiment. The *f ss* group of experiment 3 has a pattern of P_2 changes that suggests its ejaculate competitive ability changes with time after remating.

Correlation between fitness components

The only significant correlations between fitness components in the three experiments were between the number of post-remating progeny and P_2 for the *oo* group in experiment 2 ($r = -0.785$, $p < 0.05$), and the *f ss* group in experiment

TABLE 6

Analysis of variance in main effects on fitness components in experiment 3

Term	Total offspring			P_2	
	df	MS	*F*	MS	*F*
Female	1	36808.60	12.18‡	56.56	0.17
First male	3	10947.21	3.62*	1730.97	5.13†
Esterase 6	1	3631.86	1.20	2.91	0.01
X marker	1	29525.72	9.77†	3619.98	10.72†
Interaction	1	315.77	0.10	1561.35	4.62*
Second male	3	9629.86	3.19*	3566.83	10.56‡
Esterase 6	1	5819.47	1.93	93.31	0.28
X marker	1	4828.45	1.60	10526.61	31.18‡
Interaction	1	18511.74	6.12*	122.31	0.36
Within groups	60	3022.85		337.65	

Levels of significance: *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$.

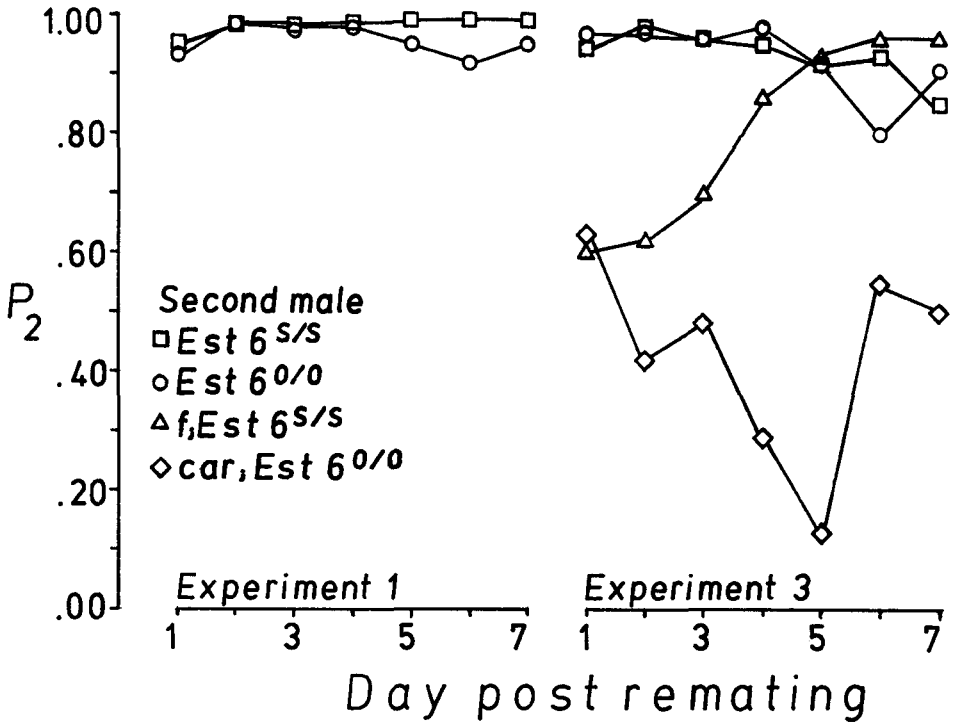

FIGURE 1.—Proportion of second-male offspring, P_2, as a function of day after remating for second-male groups of experiments 1 and 3.

3 ($r = +0.584$, $p < 0.01$). These results and the absence of significant correlations for the *ss* and *oo* groups of experiment 1 again suggest that the effects noted are due primarily to the markers used and not to EST-6 type.

DISCUSSION

Our experiments were designed to measure the effects of EST-6 on second-male ejaculate competitive ability for three models of natural mating dynamics in Drosophila. (1) The competitive influence of the first male's ejaculate is regulated by female choice of remating time. (2) Second males compete directly with first males and with their ejaculates for several females. (3) The competitive influence of the first male's ejaculate is maximized by rapid remating. In all three experiments in which second males competed only with the ejaculates of first males, we found no significant effect of EST-6 type nor of mating design on the value of P_2, as summarized in Table 7. We report a significant effect of EST-6 type on P_2 when second and first males compete directly (Table 4) and a significant effect of marker type on P_2. This EST-6 effect is consistent with our evidence for an EST-6-controlled reduction in time of remating (RICHMOND *et al.* 1980; GILBERT, RICHMOND and SHEEHAN 1981b).

The index used to compare ejaculate competitive ability, P_2, is an appropriate measure of a male fitness component (Table 6). In contrast to total productivity,

TABLE 7

Summary of P_2, proportion of second male offspring, for experiments 1, 2 and 3

	Second-male genotype†			
	ss	*oo*	*f ss*	*car oo*
Experiment 1	0.967	0.941		
	79.5 ± 1.48°	75.9 ± 2.09°		
Experiment 2*	0.941	0.961		
	75.9 ± 1.15°	78.7 ± 1.49°		
Experiment 3	0.952	0.957	0.676	0.585
	77.3 ± 4.46°	78.0 ± 2.26°	55.3 ± 5.21°	49.9 ± 5.57°

* Groups B and C.
† Differences among experiments for the *ss* and *oo* groups and between *ss* and *oo* groups are not significant: $F(5,106) = 0.02$.

P_2 was not influenced by female genotype but was determined primarily by second-male genotype. Our observation that P_2 is invariant between experiments that differ in the strength of competition presented by first-male sperm suggests that the mechanisms involved in this process may be adapted to respond to various intensities of competition. The significantly poorer competitive abilities of *car* and *f* males have been found previously (LOBASHOV 1939; LEFEVRE and JONSSON 1963) and may be a direct effect of these alleles, such as the pleiotropic effect of several marker loci on female reproductive physiology (DOBZHANSKY and HOLZ 1943; ANDERSON 1945). The significant increase in P_2 with time from remating found for *f* males (Figure 1) suggests that the competitive dysfunction affects only receptacle sperm use, as there are indications that receptacle-stored sperm predominate in fertilization during the first three days post-mating, while spermathecally stored sperm take precedence thereafter (FOWLER 1973; GILBERT, submitted).

The mechanisms of ejaculate competition in insects are generally unknown (PARKER 1970; SMITH 1979). Physical displacement of first-male sperm is accomplished with a penile "sperm hook" by second-male damselflies (WAAGE 1979), but *Drosophila melanogaster* males have no analogous structures (*e.g.*, MILLER 1950). We have disproved the hypothesis that EST-6 acts as a sperm displacing enzyme, but the pronounced and distinct effects on P_2 associated with the forked and carnation markers indicate that the mechanisms of ejaculate competition in Drosophila may be amenable to genetic analysis.

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