Sperm Precedence of Irradiated Mediterranean Fruit Fly Males (Diptera: Tephritidae)

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Abstract. Irradiated and non-irradiated Mediterranean fruit fly Ceratitis capitata (Wiedemann), males of two genotypes exhibited second male sperm precedence over the major portion of the female’s reproductive lifespan. The majority of females from polygamous copulations produced >50% of offspring from the second male. This figure is a mean number and there was great individual variation among females with values for ranging from 0.23 to 0.95. Females mated sequentially to irradiated and non-irradiated males had decreased fertility when the second male was irradiated. Females mated to irradiated males recovered fertility when remated to a non-irradiated male. These findings are of significance to sterile insect release (SIT) programs for control of the Mediterranean fruit fly where released flies will mate with wild flies and with concurrently released sterile flies. It indicates that fertility can be recovered even after a wild female mates with a released sterile male if she subsequently mates with a fertile wild male.

Key words: Mediterranean fruit fly; sterile insect release; polygamous copulations.

The wide host range of the Mediterranean fruit fly, Ceratitis capitata (Wiedemann), and the threat of its further expansion into fruit growing regions in subtropical and tropical regions make it one of the most feared agricultural pests. The sterile insect release method is the current technique of choice for eradication of introduced populations, i.e., in the continental U. S. (Cunningham et al. 1980, Mitchell and Saul 1990). High male mating competitiveness is critical for the success of this technique because sterile males must seek out and mate with wild females in order to suppress the target population (Holbrook and Fujimoto 1970, Hooper and Katiyar 1971). Several factors have been identified which may influence male mating competitiveness: (1) radiation dosage (Hooper 1972), (2) irradiation atmosphere (Ohinata et al. 1977), and (3) stage of irradiation (Ohinata et al. 1971).

McInnis (1993) found field-collected female Mediterranean fruit flies that had mated with both sterile and wild males. The effectiveness of the sterile insect release method, therefore, is influenced by the dynamics of sperm use in wild females sequentially mated to irradiated released males and wild males. If a sterile male mates with a previously mated wild female, the sperm of the sterile male will be placed in a situation in which it may have an advantage, no advantage or a disadvantage with sperm from wild males. Although irradiation decreases mating competitiveness, sperm of irradiated males that are second copulators have precedence in the offspring (Ito and Yamagishi 1989, Katiyar and Ramirez 1970). Females mated with non-irradiated males first and irradiated males second appeared to have decreased fertility when compared to once-mated females. Similarly, females mated with irradiated males first and non-irradiated males second had increased fertility when compared to once-mated females (Ito and Yamagishi 1989, Katiyar and Ramirez 1970).

Second male sperm precedence in females mated to non-irradiated males was observed in the Mediterranean fruit fly (Saul et al. 1988, Saul and McCombs 1993a) using genetic markers to assess the reproductive fitness of males and females in polygamous copulations.
Polygamous copulations could be reproductively advantageous for the second mate and the female because twice-mated females had prolonged progeny production (Saul and McCombs 1993a). Second mates of twice-mated females had sperm precedence; however, there were no significant differences between lone and second males in total progeny production.

The present study determines the effects of irradiated male copulation order on sperm precedence for individual females over the major portion of their reproductive lifespan; extending previous investigations by Causse (1970) and Katiyar and Ramirez (1970). Female fitness and genotypic effects on sperm precedence in females sequentially mated to irradiated and non-irradiated males were investigated by reciprocal copulations (Saul et al. 1988, Saul and McCombs 1993a).

**Materials and Methods**

**Rearing.** Mediterranean fruit fly strains were maintained in the Genetic Stock and Clone Center for Tephritid Fruit Flies located in a quarantine facility at the Department of Entomology, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu. Adult and larval rearing methods and diets were as described by Saul (1982) with an egg-to-egg generation time of approximately 30 d under normal laboratory conditions (23-25°C, 65-75% RH). Wax-coated cylindrical paper containers (8.5 cm diameter, 8.5 cm high, 473 ml) with fiberglass mesh screen covers were used for adult rearing and in copulation experiments.

Pupariation and eclosion from irradiated pupae were synchronized by collecting mature third instars in water for 24 h and then mixing them with vermiculite to initiate pupariation. Pupae were held at 23°C for 9 d, then sealed in air-tight plastic bags for 1 h to create hypoxic conditions. Pupae were exposed to 15 krad of gamma radiation from the cobalt-60 source at the Hawaii Research Irradiator, University of Hawaii at Manoa, Honolulu. Pupae were held at 23°C for eclosion and males were collected within 24 h to ensure virginity.

**Genetic lines.** Test females were homozygous recessive for 2 non-linked genes, white pupae (Rössler 1979) and dark pupae (w/w; dp/dp) (Rössler and Koltin 1976). Two male genotypes were used, wild-type (+/+; +/+ with brown puparia and dark pupae (+/+; dp/dp) with black puparia. Successful fertilization of a test female by a wild-type male resulted in progeny with brown puparia. In contrast, successful fertilization of a test female by a dark-pupae male resulted in progeny with black puparia.

**Testing protocols.** Adults used in copulation trials were 1 - 2 wk old unless otherwise stated. Virgin test females were aspirated singly into cages 1 d before the copulation trial. Copulation trials were initiated between 0800-0930 hours by aspiration of one virgin male into each cage and observations continued for 8 h. Copulating flies were allowed to separate naturally, then the males were removed. Copulation, defined as genital contact for at least 15 minutes (Saul and McCombs 1993a), and copulation duration were recorded. The term fertilization was used when hatch rate indicated that sperm was successfully transferred.

**Single copulations.** Twenty cages received wild-type males and twenty cages received dark-pupae males. Eggs were collected every 3 d for 30 d, beginning on the day of copulation. Eggs were removed from the vials after 24 h and counted onto 2 x 2 cm green blotter paper. The blotter paper was placed on larval media in a closed container to maintain humidity for the 3-4 d during which hatch was recorded. Females producing infertile eggs during the initial 3 egg collections were classified as unmated and discarded. Pupae were counted 21 d after egg collection.

**Single copulations with irradiated males.** The single copulation procedure was followed with modification: 1) the males were irradiated; 2) 60-120 copulation pairs were used per
trial; and 3) eggs counted onto blotter paper were placed in a humidified dish.

**Double copulations.** Sixty cages received wild-type males and 60 cages received dark-pupae males. The recopulation trial was conducted 24 h after the initial copulation trial by introducing a male of the alternate genotype. For example, if the first copulation was with a wild-type male, then the dark-pupae male was introduced into the cage for the recopulation trial. Eggs were collected, counted, and fertile eggs recorded in the same manner as the single copulations. The pupae were separated by phenotype and counted after 21 d.

**Sequential copulations with irradiated and non-irradiated males.** The procedure for sequential copulations with irradiated and non-irradiated males was the same as the double copulation procedure except one irradiated male was tested in each pair. Males 4-12 d old were used because it was very difficult to induce copulation in older irradiated males.

**Statistical techniques.** A pairwise t test or Mann-Whitney test (Campbell 1974; Independent-Samples T Test and Nonparametric Two-Independent-Samples Tests, SPSS 1993) was used for all comparisons. Proportions of second male offspring for multiple copulation sequences were transformed with the angular function (Campbell 1974) before analysis, and back-transformed means are reported.

### Results and Discussion

**Copulation experiments. Single copulations.** A total of 120 copulating pairs were used (60 copulating pairs for each of the two male genotypes). Twenty-seven females copulated with a wild-type male and 21 of these copulations resulted in fertilizations. Sixteen of the 21 mated females survived up to 30 d. Thirty-three females copulated with a dark-pupae male and 17 of these copulations resulted in fertilizations. Thirteen of the 17 mated females survived up to 30 d.

The genotype of the male had no significant effect on egg production or pupal production (Table 1). This indicates that when the males mated once, male fitness was not significantly affected by genotype. Standard practice has been to regard the proportion of pupae with a given genetic marker to be an unbiased indicator of the proportion of sperm carrying that

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Eggs</th>
<th>Pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>646.13</td>
<td>411.44</td>
</tr>
<tr>
<td>Dark-pupae</td>
<td>676.23</td>
<td>431.69</td>
</tr>
<tr>
<td>I Wild-type</td>
<td>541.73</td>
<td>—</td>
</tr>
<tr>
<td>I Dark-pupae</td>
<td>653.36</td>
<td>—</td>
</tr>
</tbody>
</table>

Standard error and sample size (number of females) are in parentheses.

Mean number of eggs are not significantly different for females mated to (1) irradiated wild-type males and irradiated dark-pupae males; (2) non-irradiated wild-type males and non-irradiated dark-pupae males; and (3) irradiated and non-irradiated males of the same genotype.
genetic marker (Saul and McCombs 1993a). The more similar two male genotypes are in fitness, the less likely that sperm precedence in double copulations will be influenced by differential larval survivorship. In multiple copulations, reciprocal crosses were done to minimize any bias caused by differential larval survivorship.

Wild-type males copulated significantly \((t\text{-test}, P = 0.005)\) longer (149.63±8.65 min, \(n = 24\)), than dark-pupae males (111.10±9.84, \(n = 21\)). The mean copulation durations for both male genotypes were approximately 2 h, which is a sufficient time period for transfer of 80-99% of the sperm (Seo et al. 1990).

**Single copulations with irradiated males.** A total of 240 copulating pairs were used in single trials with irradiated males, 180 copulating pairs for the irradiated wild-type male genotype and 60 copulating pairs for the irradiated dark-pupae male genotype. Eighteen females copulated with an irradiated wild-type male and 11 females survived up to 30 d. Sixteen females copulated with a dark-pupae male and 14 females survived up to 30 d. The copulation rate for irradiated wild-type males was 10% compared to the 27% copulation rate of dark-pupae males. Therefore, in order to get a minimum of 15 copulations for each male genotype, more pairs had to be used for irradiated wild-type males.

Male genotype had no significant effect on egg production, hatch rate, or number of infertile eggs in single copulations where the males were irradiated (Table 2, copulations A and B). Irradiation status of the male had no significant effects on egg production by the females with which they mated (Table 1). Egg hatch was observed (Table 2, copulations A and B), indicating that some fertile sperm were produced by irradiated males. However, the mean proportion of fertile eggs was < 0.1% for both male genotypes and did not interfere with the use of fertile eggs as a marker to determine fitness of non-irradiated males in sequential copulations.

Irradiated dark-pupae males copulated significantly \((t\text{-test}, P = 0.038)\) longer (178.20±20.61 min, \(n = 15\)) than irradiated wild-type males (120.67±17.19, \(n = 18\)). The mean copulation duration for irradiated dark-pupae males was significantly longer than the duration for untreated dark-pupae males \((t\text{-test}, P = 0.008)\). The copulation duration of irradiated wild-type males was not significantly different from that of untreated wild-type males.

**Double copulations.** Approximately 300 copulating pairs were used for the copulation sequence wild-type male first and dark-pupae male second. One hundred and six females copulated with a wild-type male for the first copulation, and 44 of these copulated again after 24 h with a dark-pupae male. Five copulations were with the wild-type first male only, five copulations were with the dark pupae second male only, and twelve copulations were with both the first and second male. All of these females survived over the 30 d test period.

Approximately 480 copulation pairs were used for the copulation sequence dark-pupae male first and wild type male second. Three hundred twenty-five females copulated with a dark-pupae male for the first copulation. A total of 42 females copulated again after 24 h with a wild-type male. Paternity, based on the genotype of the pupae, indicated that two copulations resulted in fertilization by the dark-pupae first male only, seven fertilizations were by the wild-type second male only, and four fertilizations were with both the first and second male. All of these females survived for 30 d.

A limitation of any type of marker used to trace paternity in multiple mating experiments is the inability to detect total sperm precedence of a male. That is, in cases where two males transfer sperm successfully but only one male successfully fertilizes the eggs. There were more double fertilizations in the copulation sequence wild-type male first and dark-pupae male second than the reciprocal cross, as indicated by the pupa color genetic markers. We were unable to differentiate between copulations in which two males transferred sperm successfully but one male had total sperm precedence and copulations in which only one
Table 2. Mean number of eggs, fertile eggs, and infertile eggs from females copulating singly to irradiated (I) males and of females of multiple copulations

<table>
<thead>
<tr>
<th>Copulation sequence</th>
<th>Eggs(^a)</th>
<th>Fertile eggs(^b)</th>
<th>Infertile eggs(^c)</th>
<th>Pupae(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Dark pupae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. I Wild-type (^e)</td>
<td>541.73 (56.34,11)</td>
<td>0.39 (0.20, 11)</td>
<td>541.36 (56.24, 11)</td>
<td></td>
</tr>
<tr>
<td>B. I Dark-pupae (^e)</td>
<td>653.36 (32.67,14)</td>
<td>1.14 (0.31, 14)</td>
<td>652.21 (32.50, 14)</td>
<td></td>
</tr>
<tr>
<td>C. Wild-type ⇒ dark-pupae</td>
<td>750.83 (47.10,12)</td>
<td>646.67 (46.35,12)</td>
<td></td>
<td>282.42 (44.09,12)</td>
</tr>
<tr>
<td>D. Dark-pupae ⇒ wild-type</td>
<td>493.50 (78.64,4)</td>
<td>381.50 (125.17,4)</td>
<td></td>
<td>222.25 (105.61,4)</td>
</tr>
<tr>
<td>E. I Wild-type ⇒ dark-pupae</td>
<td>693.60 (47.01,15)</td>
<td>351.0 (62.40,15)</td>
<td>342.53 (47.59,15)</td>
<td></td>
</tr>
<tr>
<td>F. I Dark-pupae ⇒ wild-type</td>
<td>697.17 (47.85,12)</td>
<td>420.00 (83.14,12)</td>
<td>277.17 (87.40,12)</td>
<td></td>
</tr>
<tr>
<td>G. Wild-type ⇒ I dark-pupae</td>
<td>594.64 (63.34,11)</td>
<td>99.00 (53.06,11)</td>
<td>495.64 (80.29,11)</td>
<td></td>
</tr>
<tr>
<td>H. Dark-pupae ⇒ I wild-type</td>
<td>637.78 (56.72,9)</td>
<td>3.00 (0.65,9)</td>
<td>634.78 (56.99,9)</td>
<td></td>
</tr>
</tbody>
</table>

Standard error and sample size (number of females) are in parentheses.

\(^a\) Mean number of eggs is not significantly different between sequential copulation sequences.

\(^b\) Significant differences between E and G (Mann-Whitney test, \(P = 0.0039\)), E and H (\(t\) test, \(P = 0.000\)), F and G (Mann-Whitney test, \(P = 0.0104\)), and F and H (\(t\) test, \(P = 0.000\)).

\(^c\) Mean is significantly different for E and H (\(t\) test, \(P = 0.001\)) and F and H (\(t\) test, \(P = 0.003\)).

\(^d\) Mean number of wild-type and dark pupae are not significantly different within each copulation sequence.

\(^e\) Mean number of eggs, infertile eggs, and fertile eggs are not significantly different for single copulations.
male successfully transferred sperm.

The copulation sequence wild-type male first and dark-pupae male second produced significantly more eggs and fertile eggs than the copulation sequence dark-pupae male first and wild-type male second (Table 2, copulations C and D). These results were unexpected because egg production is normally a function of factors determined by the female. There was no significant difference in total pupae or in numbers of wild-type pupae and dark-pupae between copulation sequences (Table 2, copulations C and D). This indicates that copulation sequence may influence egg production, however nutrition and other factors should not be rejected as possible causes for the differential egg production.

**Sequential copulations with irradiated and non-irradiated males.** Approximately 240 copulating pairs were used for the copulation sequence irradiated wild-type male first and non-irradiated dark-pupae male second. Seventy-six females copulated with an irradiated wild-type male for the first copulation and 30 of these copulated again after 24 h with a dark-pupae male. Twenty-nine females had eggs that hatched, indicating fertilization with the non-irradiated dark pupae male. Fifteen of the 29 females survived over 30 d.

Approximately 200 copulating pairs were used for the copulation sequence irradiated dark-pupae male first and non-irradiated wild-type male second. Seventy-four females copulated with an irradiated dark pupae male for the first copulation. Twenty of these copulated again after 24 h with a wild-type male. Sixteen females had eggs that hatched, indicating fertilization with the non-irradiated wild-type males. Twelve of the 16 females survived over 30d.

Approximately 699 copulating pairs were used for the copulation sequence non-irradiated wild-type male first and irradiated dark-pupae male second. Three hundred and fourteen females copulated with a wild-type male for the first copulation. Of these, 19 copulated with an irradiated dark pupae male after 24 h. Thirteen of these females had eggs that hatched which indicated fertilization with the non-irradiated wild-type males. Eleven of the 13 females survived over 30 d.

Approximately 714 copulating pairs were used for the copulation sequence non-irradiated wild-type male first and irradiated dark pupae male second. Three hundred and fifteen females copulated with a dark pupae male for the first copulation. Of these, 20 subsequently copulated with an irradiated wild-type male after 24 h. Thirteen of these females had eggs that hatched which indicated fertilization with the non-irradiated dark pupae males. Nine of the 13 females survived over 30 d.

There were no significant differences in egg production between the four possible copulation sequences (Table 2, E–H). The hatch rate from the copulation sequence dark-pupae male first and irradiated wild-type male second was extremely low, and consequently the number of infertile eggs was very high, significantly higher than copulation sequences where the first male was irradiated (Table 2). There are two main possible explanations: 1) strong second male precedence occurred and fertilization was by the second male only, or, 2) only a small quantity of sperm was transferred. The fertilization success rate for the normal double copulation sequence dark-pupae male first and wild-type male second indicated that in most cases, the second male was the only copulator or extreme sperm precedence occurred.

Females mated to an irradiated male first and a non-irradiated male second had significantly more fertile eggs than females in the reciprocal copulations, E vs. G and F vs. H (Table 2). These data were consistent with reports of Katiyar and Ramirez (1970) and suggests second male precedence for irradiated and non-irradiated males mated sequentially to a female.

**Sperm precedence. Proportion of infertile eggs.** There was no significant difference in the proportion of infertile eggs produced from copulation sequences A and B (Table 3).
Table 3. Infertile eggs and proportion of second male offspring over 3 and 30 d for the double copulations and sequential copulations with irradiated (I) and non-irradiated males.

<table>
<thead>
<tr>
<th>Copulation sequence</th>
<th>P₂</th>
<th></th>
<th>Proportion infertile eggs b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d</td>
<td>30 d</td>
<td>3 d</td>
</tr>
<tr>
<td>A. Wild-type ⇒ dark-pupae</td>
<td>0.59 (0.004,18)</td>
<td>0.51 (0.008,12)</td>
<td>0.08(0.002,18)</td>
</tr>
<tr>
<td>B. Dark-pupae ⇒ wild-type</td>
<td>0.85 (0.006,12)</td>
<td>0.74 (0.032,4)</td>
<td>0.14(0.008,12)</td>
</tr>
<tr>
<td>C. I wild-type ⇒ dark-pupae</td>
<td>0.58 (0.004,30)</td>
<td>0.46 (0.008,15)</td>
<td>0.54 (0.001,15)</td>
</tr>
<tr>
<td>D. I dark-pupae ⇒ wild-type</td>
<td>0.67 (0.019,18)</td>
<td>0.61 (0.020,12)</td>
<td>0.39 (0.020,12)</td>
</tr>
<tr>
<td>E. Wild-type ⇒ I dark-pupae</td>
<td>0.82 (0.012,16)</td>
<td>0.92 (0.016,11)</td>
<td>0.92 (0.016,11)</td>
</tr>
<tr>
<td>F. Dark-pupae ⇒ I wild-type</td>
<td>0.98 (0.004,19)</td>
<td>1.00 (0.000,9)</td>
<td>1.00 (0.000,9)</td>
</tr>
</tbody>
</table>

a Means for proportions after 3 d and 30 d are not significantly different for A (t test, $P = 0.426$); B (t test, $P = 0.410$); C (t test, $P = 0.315$); D (t test, $P = 0.792$); E (t test, $P = 0.410$) and F (Mann-Whitney test, $P = 0.4554$).

b Proportion of infertile eggs over 3 and 30 d were not significantly different for A (t test, $P = 0.130$) and B (Mann-Whitney test, $P = 0.1822$). Proportions are significantly different for C and F (t test, $P = 0.000$) and D and E (t test, $P = 0.005$). Means for proportions A and B were not significantly different.
Planned comparisons were made between reciprocal copulation sequences with the same male genotype irradiated. Copulation sequence E (irradiated wild-type male followed by dark-pupae male) had a significantly higher proportion of infertile eggs than copulation sequence D (dark-pupae male followed by wild-type male) (Table 3). Similarly, copulation sequence F (irradiated dark-pupae male followed by wild-type male) had a significantly higher proportion of infertile eggs than copulation sequence C (wild-type male followed by dark-pupae male). The proportion of infertile eggs increased when the second male was irradiated, indicating precedence of second male sperm of both genotypes. The results were consistent with observations of Katiyar and Ramirez (1970) that females mated to irradiated second males had decreased fertility.

The egg infertility rate of the sequential copulations with irradiated and non-irradiated males consisted of the normal infertility rate (Table 3, A and B) and the infertility rate that resulted from eggs fertilized by irradiated sperm. Therefore, if there was second male precedence, females who mated with irradiated males second would have a higher proportion of infertile eggs than females who mated with the irradiated male first. This was observed in comparisons of copulation sequences C and F and sequences D and E (Table 3).

**Fitness of non-irradiated males in sequential copulations with irradiated males.** In sequential copulations with irradiated and non-irradiated males, second male sperm precedence for the non-irradiated males of both genotypes was observed over 3 d and 30 d. Copulation sequence A (irradiated wild-type male followed by dark-pupae male) produced significantly more fertile eggs over 3 d than copulation sequence D (dark-pupae male followed by irradiated wild-type male) (Table 4). Similarly, copulation sequence B (irradiated dark-pupae male) produced significantly more fertile eggs than copulation sequence C (wild-type male followed by irradiated dark-pupae male). Over 30 d, copulation sequence A produced significantly more fertile eggs than copulation sequence D, and copulation sequence B produced significantly more fertile eggs than copulation sequence C (Table 4).

Male genotype as well as copulation order could affect male sperm precedence (Saul and McCombs 1993a; Prout and Bundgaard 1977). The number of fertile eggs for copulation sequence D (dark-pupae male followed by irradiated wild-type male) (Table 4) was significantly lower than that of copulation sequence C (wild-type male followed by irradiated dark-pupae male) over 3 d and 30 d. The proportion of pupae with the genotype of the second male copulating for the copulation sequence B (dark-pupae male first and wild-type male second) was very high, indicating that the second male may have been the only fertilizer or there was extreme sperm precedence (Table 3).

**Male fitness in single and double copulations.** For both male genotypes, the second copulator produced significantly more pupae than the first copulator over 3 d (Table 5). The number of pupae was reduced, however, compared to the case where the male was the lone copulator. These results are consistent with Saul and McCombs (1993a) who found that the second copulator could decrease the fitness of the first copulator in double copulations. The magnitude of the drop in fitness of the first copulator in double matings compared to monogamous matings is a function of the male genotype. Dark-pupae second copulators produced significantly less pupae than lone copulators, but wild-type pupae production was lower, but not significantly different from that of lone copulators.

The results over 30 d were difficult to explain since the decrease in sample size over the 30 d period resulted in an increase in the variance for both male genotypes. In general there were more pupae produced over 30 d than 3 d for all cases. The difference was not significant for numbers of pupae produced by wild-type males over 30 d as lone, first, and second copulator (Table 5) although the lone copulator number was highest. For the dark-pupae case the difference was significant and the lone copulator produced more pupae than the first and second copulator.
Table 4. Fitness of non-irradiated males over 3 and 30 in sequential copulations to females with irradiated (I) males

<table>
<thead>
<tr>
<th>Copulation sequence</th>
<th>Fertile eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d</td>
</tr>
<tr>
<td>A. I Wild-type ⇒ dark-pupae</td>
<td>85.67 (9.33, 30)</td>
</tr>
<tr>
<td>B. I Dark-pupae ⇒ wild-type</td>
<td>80.83 (13.36, 18)</td>
</tr>
<tr>
<td>C. Wild-type ⇒ I dark-pupae</td>
<td>32.53 (10.41, 17)</td>
</tr>
<tr>
<td>D. Dark-pupae ⇒ I wild-type</td>
<td>6.95 (5.62, 19)</td>
</tr>
</tbody>
</table>

Standard error and sample size (number of females) are in parentheses.

a Mean number of eggs hatched from copulation sequences A and D (t test, \( P = 0.000 \)) and copulation sequences B and C (Mann-Whitney test, \( P = 0.0104 \)) are significantly different. The other copulation sequences were not compared.

b Mean number of eggs hatched from copulation sequences A and D (Mann-Whitney test, \( P = 0.0000 \)) and copulation sequences B and C (t test, \( P = 0.008 \)) are significantly different. The other copulation sequences were not compared.

**Contribution of the second male.** Offspring were measured as the proportion of fertile eggs for copulations in which the second male was non-irradiated and as the proportion of infertile eggs for copulations in which the second male was irradiated. There was no significant change in the proportion of offspring from the second male, \( P_2 \), over time (Table 3). These results were consistent with those of Saul and McCombs (1993a) who found that \( P_2 \) did not change significantly from 10 to 30 d.

\( P_2 \) values calculated for each individual female ranged from 0 to 1. A \( P_2 \) value of 0.5 indicated no sperm precedence, and a \( P_2 \) value above 0.5 indicated a greater contribution of the second male over the first male. For the copulation sequence wild-type male first and dark-pupae male second, \( P_2 \) values for the 18 females range from 0.33-0.95 over 3 d. \( P_2 \) values for the 12 females remaining at 30 d ranged from 0.24-1.00. For the copulation sequence dark-pupae male first and wild-type male second, the \( P_2 \) range for 12 females over 3 d was 0.21-0.99, and \( P_2 \) for the 4 remaining females at 30 d was 0.34-0.98.

The wide ranges in \( P_2 \) values for both copulation sequences indicated a wide variation between the males but variation between the two copulation sequences also differed. The copulation sequence wild-type male first and dark-pupae male second had a full range of precedence cases. The mean \( P_2 \) value was 0.59 over 3 d and 0.51 over 30 d (Table 3). The reciprocal cross had only one case over 3 d and 30d which displayed precedence of the first copulater; the rest of the cases displayed precedence of the second copulater. The mean \( P_2 \) value was 0.85 over 3 d and 0.74 over 30 d (Table 3). Second male sperm precedence and an advantage inherent in the wild-type genotype may have contributed to its reproductive advantage over dark-pupae males. The data indicated that male genotype, copulation order, and genotypic differences could affect the variation in precedence between the males of a genotype and consequently affect genotype fitness.
Wide variation in $P_2$ values between individual female offspring from two males is common in many insects (Lewis and Austad 1990). In the Mediterranean fruit fly, second male sperm precedence may not always occur in offspring from individual females mated to 2 males, instead second male sperm precedence appears to be a trend in a sample population. The data suggested that sperm mixing occurs which somehow favors the second male sperm in most cases. The exact mechanism is unknown.

In normal double copulations, the proportion of infertile eggs was lower than 0.50 during early egg production and did not change significantly with time (Table 3). In sequential copulations with irradiated and non-irradiated males, the proportion of infertile eggs was higher than 0.50 during early egg production and did not change significantly over time. This suggests that irradiated sperm made a significant contribution during early egg production, and with time, the effects of infertility and irradiated male sperm contribution may have influenced the number of infertile eggs. The magnitude of the influence of infertility and contribution of the irradiated male to the number of infertile eggs is unknown.

**Female fitness.** Fitness of single-mated females and double-mated females was measured as the mean number of pupae produced over 30 d and over the last 9 d (Table 6). Pupae production from days 21–30 for females copulating with a wild-type male first and a dark-pupae male second was significantly greater than for either single strain copulation. However, there was no significant difference between pupae production from single copulations and double copulations with dark-pupae male first and wild-type male second, indicating that male genotype may play a role in female fitness. Pyle and Gromko (1978) and Gromko (1992) indicated that multiple-mated female *D. melanogaster* have a longer reproductive period than single-mated females. Multiply-mated females of the Mediterranean fruit fly have a longer reproductive period (Saul and McCombs 1993a) which, in the absence of larval or pupal diapause (Back and Pemberton 1918, Bodenheimer 1951), may allow a population to survive during periods of environmental stress.

Copulation sequences with the irradiated male first produced significantly more fertile eggs over 30 d than copulation sequences with the irradiated male second (Table 4). The results indicated that even if the female mated with an irradiated male, she could recover fertility by copulation with a non-irradiated male. However, female fitness dropped when she mated a second time to an irradiated male. The second copulation affected female fitness more than the first copulation, which was evidence of second male sperm precedence.

Sequential copulations with irradiated and non-irradiated males indicated that second male sperm precedence in two male genotypes of the Mediterranean fruit fly remained consistent over the major portion of the female’s reproductive lifespan. Both irradiated and non-irradiated males exhibited second male sperm precedence for both male genotypes. Copulation order of the irradiated male and male genotype affected the reproductive contribution of a male in polygamous copulations.

Variation in sperm precedence between individual males should be investigated in conjunction with the physiological basis for sperm precedence. Second male sperm precedence does not always occur for individual females, but the mean second male contribution, $P_2$, is greater than 0.5. Sperm mixing seems to occur but the exact mechanism is unknown. Understanding the mechanism of sperm precedence may be useful for modifying the sterile insect release method to increase its efficiency, i.e., if the mechanism for sperm precedence is associated with direct competition between sperm from two males, then a genotype with competitive sperm should be used for the sterile insect release method.

These findings are of significance to sterile insect release (SIT) programs for control of the Mediterranean fruit fly where released flies will mate with both wild flies and with concurrently released sterile flies. The results indicate that females mated sequentially to irradiated and non-irradiated males had decreased fitness when mated to a non-irradiated
<table>
<thead>
<tr>
<th>Copulation</th>
<th>Male</th>
<th>Wild-type</th>
<th></th>
<th>Dark-pupae</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 d&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 d</td>
<td>3 d&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 d&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Single</td>
<td>Lone</td>
<td>100.00 (12.13,24)</td>
<td>411.44 (52.72,16)</td>
<td>117.70 (17.15,20)</td>
<td>431.69 (56.09,13)</td>
</tr>
<tr>
<td>Double</td>
<td>First</td>
<td>39.50 (6.39,18)</td>
<td>282.42 (44.09,12)</td>
<td>12.25 (3.28,12)</td>
<td>123.50 (66.98,4)</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>75.58 (11.36,12)</td>
<td>222.25 (105.61,4)</td>
<td>64.56 (10.43,18)</td>
<td>275.42 (47.72,12)</td>
</tr>
</tbody>
</table>

Standard error and sample size (number of females) are in parentheses.
<sup>a</sup>Wild-type lone copulater is significantly different from wild-type first copulater (t test, \( P = 0.000 \)) and wild-type second copulater is significantly different from wild-type first copulater.

<sup>b</sup>Dark-pupae lone copulater is significantly different from dark-pupae first copulater (t test, \( P = 0.000 \)) and dark-pupae second copulater (t test, \( P = 0.013 \)). Dark pupae first copulater and second copulater are significantly different (t test, \( P = 0.000 \)).

<sup>c</sup>Dark-pupae lone copulater is significantly different from dark-pupae first copulater (t test, \( P = 0.013 \)) and dark-pupae second copulater (t test, \( P = 0.046 \)). Dark pupae first copulater and second copulater are not significantly different.
Table 6. Fitness of single- and double-mated females.

<table>
<thead>
<tr>
<th>Copulation type</th>
<th>Male sequence</th>
<th>No. Pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cumulative&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Single</td>
<td>A. Wild-type</td>
<td>411.44 (52.72,16)</td>
</tr>
<tr>
<td></td>
<td>B. Dark-pupae</td>
<td>431.69 (56.09,13)</td>
</tr>
<tr>
<td>Double</td>
<td>C. Wild-type ⇒ dark pupae</td>
<td>557.83 (41.89,12)</td>
</tr>
<tr>
<td></td>
<td>D. Dark pupae ⇒ wild-type</td>
<td>345.74 (123.66,4)</td>
</tr>
</tbody>
</table>

Values are back-transformed means. Standard errors and sample sizes (number of females) are in parentheses.

<sup>a</sup>Cumulative mean number of pupae from copulations A and C are significantly different ($t$ test, $P = 0.049$).

<sup>b</sup>Mean number of pupae are significantly different from copulations A and C ($t$ test, $P = 0.029$) and copulations B and C ($t$ test, $P = 0.040$).

male first and then an irradiated male. However in the reciprocal cross, females mated to an irradiated male could recover fertility by copulation with a fertile male. This indicates that fertility can be recovered even after a wild female mates with a released sterile male if she subsequently mates with a fertile wild male.

In terms of the sterile insect release method, it would be advantageous for the second copulator to be a released sterile male not a wild male. Thus the release of a Mediterranean fruit fly strain with a high success rate in copulating with previously-mated females could improve the effectiveness of sterile insect release programs (Saul and McCombs 1993b).

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