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THE GENETIC AND ENVIRONMENTAL EFFECTS ON HYBRIDIZATION BETWEEN DROSOPHILA MELANOGASTER AND D. SIMULANS

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THE GENETIC AND ENVIRONMENTAL EFFECTS ON HYBRIDIZATION BETWEEN DROSOPHILA MELANOGASTER AND D. SIMULANS

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CHAPTER I

INTRODUCTION

It is well known that some related species which are distinct, and rarely if ever hybridize in nature, will mate and have offspring under laboratory conditions (Dobzhansky, 1964). This is the case for the sibling species Drosophila melanogaster and D. simulans (Diptera, Drosophilidae, subgenus Sophophora). The extensive growth of research on mating behavior in Drosophila has led to a greater understanding of the genetic and non-genetic influences on mating behavior. The purpose of this research is to investigate certain genetic and environmental effects on the hybridization of these two species. Since their isolation in the laboratory is entirely behavioral or sexual (though reinforced by hybrid sterility), rather than mechanical, temporal, or ecological, it is appropriate to consider the genetic and environmental influence on both the mating behavior of the two species and their sexual isolation. Behavioral and sexual isolation are often used interchangeably (Levine, 1969) and refer here to isolating mechanisms which function after the flies come together but before sperm transfer occurs. Mating behavior and sexual isolation will be considered separately, however, for two reasons: (1) although sexual isolation studies draw upon

mating behavior studies and vice versa, basic mating behavior studies are most often concerned with intraspecific matings and sexual isolation studies are often concerned with interspecific crosses and the particular factors affecting their success; (2) there is some evidence (Kessler, 1966) that intraspecific mating and interspecific mating are determined, at least in part, by different genes.

A. Environmental and Other Non-genetic Influence on Mating Behavior

1. Odor

Sturtevant (1915) reported that D. melanogaster mated more rapidly in vials which had held copulating flies than in clean vials, and Jacobs (1960) claims to have repeated the experiments and confirmed Sturtevant's results, though he furnished no data. Ewing and Manning (1963), however, found no differences in mating after exposing flies to air which had been drawn over courting and copulating flies, nor was there any difference in mating speed in clean containers and those which had held mating flies.

Jacobs (1960) investigated the effects of odors of nearby flies and found that melanogaster males treated with female juices received more precopulatory motions from males than did the untreated males. The possible effect of scent was confirmed by the fact that males made no wing extensions toward male heads while in the dark and very few while in the

light, although a larger number of wing extensions were directed toward female heads in each case.

2. Age and Isolation from the Opposite Sex

Bastock and Manning (1955) reported that melanogaster males isolated since eclosion have a shorter lag time before beginning to court a virgin female, and Barker (1962a) found that wild type females, normally resistant to courtship by yellow-bodied males, are inseminated more often by yellow males if the flies (of both sexes) are a week old than if they are only a few hours old. Hoenigsberg and Santibanez (1960) state that uniform age may be important for obtaining uniform courtship behavior in melanogaster since flies of different ages may have different sensory thresholds.

3. Light

The effect of light on mating behavior is quite variable. Spieth and Hsu (1950) report that melanogaster mates readily in light or dark but that simulans' mating frequency is greatly inhibited in the dark; D. subobscura does not mate at all in the dark (Petit and Ehrman, 1969). Darkness does not cause uniform reduction of mating within a species, however, since homozygous ebony strains (of melanogaster) have an advantage over wild type in the dark (Petit and Ehrman, 1969).

4. Previous Copulations

Previous copulations have a distinct effect on female receptivity. A melanogaster female just inseminated will not accept other males for hours (Bastock and Manning, 1955) or even days (Bastock and Manning, 1955; Knight, Robertson, and Waddington, 1956; Manning, 1967a), although one can get multiple matings if the flies are crowded, since the females cannot avoid the males and are "raped" (Manning, 1967a).

5. "On" and "Off" Days (or Variations in Female Receptivity) and Diurnal Rhythm

It is often noted (Manning, 1961) that Drosophila have "on" and "off" days for courtship, often affecting different lines in parallel fashion, due to some common environmental factor, the nature of which is still obscure. Biddle (1932) reports that in some cases of crosses between melanogaster females and simulans males all matings set up (five or six) are successful. At other times up to 50 unsuccessful attempts may be made.

A major factor in the determination of mating success is female receptivity, which increases following eclosion to a peak three days later (for both melanogaster and simulans), but significant variation in female receptivity is found between experiments on different days (Manning, 1959a). Mayr (1946) states that sexual activity in Drosophila is unpredictable,

especially in well-aged flies, being high on certain days, and low on others, even though all environmental conditions are seemingly identical. Mayr also reports (1946) that sexual activity is higher in the morning and evening than in the middle of the day. Kvelland (1965a), however, found that males stored for either two hours or three days (before being mated) show greater mating activity in the first hour of a twelve hour period (8 AM to 8 PM) than in any other interval and that, except for the first hour interval, the male mating activity was the same in all intervals of the day (although only those within the time period cited were studied).

6. Time Available for Mating

The time interval available is important, since more flies are inseminated during longer time intervals than during shorter ones (Barker, 1962a). Male melanogaster, however, which court fertilized (unreceptive) females or other males may completely cease to court, even though females are still available (Bastock and Manning, 1955).

7. Sound and Air Currents

Wing vibration in Drosophila melanogaster is accompanied by a train of sound pulses (Shorey, 1962), which provide stimuli rendering females sexually receptive (Bennet-Clark and Ewing, 1967). Bennet-Clark and Ewing also found (1967) that adding sound (recorded during the wing vibrations of wild type

males) or air currents or both significantly facilitated the courtship of wingless melanogaster males. Many of the Drosophila species studied have a characteristic courtship song, and in each species the wings can be seen to vibrate while this sound is produced (Ewing and Bennet-Clark, 1968). It has also been shown that the courtship sounds of Drosophila melanogaster are produced by isolated wing beats whose character is similar to those of normal flight (Bennet-Clark and Ewing, 1968).

8. Temperature

Ehrman (1966) found that D. pseudoobscura males homozygous for the Arrowhead inversion sequence (Ar/Ar) are more successful in mating with Ar/Ar females if raised at 16° than if raised at 25° (C.). It should be noted that the Arrowhead inversion is more frequent in northern regions (Oregon) than in the south (California) (Dobzhansky and Epling, 1944). Kvelland (1965) investigated temperature effects and found that a treatment of 0° C. for 30 minutes has no effect on mating activity of zero to two hour old males (melanogaster) but causes a reduction in mating activity of three day old males.

9. Nutrition

Possible nutritional and other effects were indicated when Spiess and Langer (1964) showed that the mating speed of heterokaryotypes (for the wild type and Klamath gene sequences)

depends on whether they were reared in vials or cages. Rendel (1951) stated that nutritional states affect mating behavior but provided no supporting evidence.

10. Numbers and Ratios of Flies

The numbers and ratios of flies of the two sexes influence mating behavior. Morpurgo and Nicoletti (1955) found that the selective mating (favoring wild type) which existed when wild type and white-eyed males (one of each) were with one female (of either type) disappeared when the sex ratio was 1:1. Kaul and Parsons (1966) found that mating speed is greater with three males and one female than with one male and three females (in which case the male has no competition). Barker (1962a) showed that the isolation between yellow and wild strains decreased as the number of flies per container increased. Petit (1958) showed that small variations in density (doubling or halving the total number present) have no influence on sexual selection, but that ten-fold variations have appreciable effects (at least when the competing males are white-eyed and wild type). That is, the rare type male has a mating advantage at high densities but not at low.

This mating advantage bestowed upon males which are confined with another, but more numerous, type of male has been called the "advantage of being rare" by Petit and Ehrman (1969) and has wide application. Some of the examples they cite are as follows: (1) Bar gene (B) populations and white gene (w)

populations in melanogaster; (2) in D. pseudoobscura for strains with different chromosomes, strains of different geographic origins, mutant and wild type strains, stocks selected for positive and negative geotaxis, and stocks reared at different temperatures; (3) for various combinations of flies from D. persimilis, tropicalis, willistoni, and equinoxialis. The cues involved here are obscure (Petit and Ehrman, 1969), but if a single sheet of cheesecloth is all that separates two populations, one with a 5:20 ratio (of males of two certain stocks) and one with a 15:0 ratio (of males of the same two stocks), there is no increase in mating success for the rare males in the container with the 5:20 ratio (Ehrman, 1966). This suggested that odors, substrate, or airborne vibrations are involved (Ehrman, 1966; Petit and Ehrman, 1969). Similarly, Ehrman found (1970) that if equal numbers of males of two particular stocks are placed in the one container and large numbers of one of those types of male are placed in the other container (both separated by only cheesecloth), the "artificially rare" males in the first chamber now have a mating advantage which did not exist when the two types of males were present in equal numbers without an adjacent container of flies. However, mating was again at random in the chamber with equal numbers of the two stocks if even less than one centimeter of space, between two taut layers of coarse cheese cloth, separated the two chambers. In a possible reevaluation of the cues involved in experiments in which cheesecloth separated two populations, Ehrman states

that these last results may indicate the importance of physical contact (touch) to discriminating females when presented with a choice of males.

11. Etherization

Streisinger (1948) showed that males display much less sexual isolation with etherized females of other species. For example, melanogaster males with etherized melanogaster and persimilis females inseminate the females much more at random than when the flies are awake. The same held true for pseudoobscura males with etherized pseudoobscura and persimilis females.

B. Environmental and Other Non-genetic Influences on Hybridization (Sexual Isolation)

1. Age

With regard to environmental and other non-genetic factors which might more directly influence hybridization between melanogaster and simulans, age is of considerable importance. Manning (1959b) found that the percentages of three day old male melanogaster showing courtship to one, two, three, and four day old simulans females were 83, 87, 45, and eight, respectively. Barker (1967) agrees that the age of the flies influences the success of mating but found (1962b) that sexual

isolation is higher for young flies (of both sexes). Pontecorvo (1942) found that the cross melanogaster females x simulans males proceeds readily if females are not more than two days old, but with difficulty if the females are four or more days old. Mayr and Dobzhansky (1945) found that aging males in isolation increases interspecific isolation for D. persimilis males (with pseudoobscura females), has no effect on pseudoobscura males (with persimilis females), and gives different results for interstrain matings of D. prosaltans. Dobzhansky and Koller (1938) had earlier shown reduced isolation (in both directions) between D. miranda and pseudoobscura when males are isolated after eclosion.

2. Numbers and Ratios of Flies

Some work has been done on the effects of numbers of flies and the sex ratio on mating behavior between melanogaster and simulans. Morgan (1929) found that small mass cultures yield more matings than pair matings (for simulans females with melanogaster males), and Barker (1962b) agreed that facilitation may be involved in small mass matings, with one courtship stimulating other males to activity. Barker (1967) also found that more inseminations occurred in bottles with more flies and that as the proportion of males rises (from 1:5 to 1:1 to 5:1), the percentage of females inseminated increases.

3. Presence of Other Flies

The presence of other flies in the immediate vicinity can have major effects on the success of crossing. Manning (1967b) stated that melanogaster males which don't normally persist in courting mature female simulans will do so if they have just tapped and courted a melanogaster female. Levine and Dobzhansky (1945) demonstrated that isolation between D. pseudoobscura and persimilis is less if both types of female are present with one type of male than if only one type of female is confined with foreign males; however, the results were just the opposite for strains of D. prosaltans. Mayr and Dobzhansky (1945) pro-conditioned one group of pseudoobscura and persimilis males with an excess of their own females and counter-conditioned another group with foreign females. When the two groups of males were then used in male choice experiments (one type of male confined with conspecific and heterospecific females), both pro- and counter-conditioned pseudoobscura males showed greater isolation than the controls (but the experiments were poorly controlled, according to the authors' own admission); counter-conditioned persimilis males showed a significantly decreased isolation.

4. Mixed Cultures and Temperatures

Attempts have been made to alter the isolation between two Drosophila species by rearing them together. Manning (1959b)

reported that all attempts to influence sexual isolation between melanogaster and simulans by rearing them together have so far failed. Mayr and Dobzhansky (1945) reared pseudoobscura and persimilis together. When pseudoobscura males were placed with pseudoobscura and persimilis females, there was increased isolation when flies (of both sexes) from mixed cultures were used. With persimilis males, different results were obtained at different times of the year. As Mayr and Dobzhansky pointed out, it would be of adaptive value for isolation to increase when two closely related species grow up together in nature, so that their adults would be less likely to mate and waste gametes in the production of sterile or semi-sterile hybrids. Mayr and Dobzhansky also discovered, in that same series of experiments, that the isolation is weakened at lower temperatures.

5. Female Receptivity

Much of the mating behavior of Drosophila females is influenced by physiological receptivity (Manning, 1967a). The receptivity of melanogaster and simulans females toward their own males rises to day three (after eclosion) and then slowly declines. However, the success of foreign males with these females shows a great decline between one and three days of female age. The increased success of foreign males with young females may not be due, then, to greater receptivity but to either less discrimination by young females against foreign males or differential courting by foreign males of the different

female age groups (Manning, 1959b). Pontecorvo (1942) found that once a young melanogaster female mates with a simulans male, successive cross-matings occur till old age, as though the mating reaction of the young female is not yet fully determined but still liable to conditioning by foreign males. In light of the many environmental influences on mating behavior, one may agree with Spiess (1970) that it may not be far from the truth to state that every conceivable factor that has an effect on growth and development of Drosophila must have some influence on mating behavior.

C. Genetic Influences on Mating Behavior and Hybridization

Studies of this sort have generally proceeded along three lines: single gene effects, differences between strains or stocks (of presumably different genetic makeup), and effects of selection for changed mating behavior (Manning, 1967b). However, possible cytoplasmic effects on mating behavior have also been reported and will be considered under a separate heading.

1. Single gene effects

In considering single gene effects, Manning (1965, 1967b) suggests that very few genes are neutral in their effect on mating success, and that almost any genetic change causes

quantitative behavioral changes. Williams and Reed (1944) state that single gene mutations generally produce manifold effects, and Dilger (1962) considers that because animals are highly integrated organisms, any gene substitution is almost bound to have secondary effects, sometimes far reaching. Since behavior represents the most complete integration of all body systems, almost any change in genotype will cause a discernible effect on some aspect of behavior (Ewing and Manning, 1967). Parsons (1964) found that the genotype has an extremely important influence on the time flies take to mate and considers (1967) that although some loci are not involved directly in mating behavior, it is likely that many loci have pleiotropic effects involving mating behavior.

To consider some of the single gene effects on mating behavior, Bastock and Manning (1955) and Bastock (1956) found that melanogaster males hemizygous for the sex-linked recessive gene yellow (body) not only take longer to begin courting, but also have to court females longer than wild type males, indicating a less vigorous or stimulating courtship. Geer and Green (1962) found that mating success improved with increasing eye pigmentation in a series of alleles at the sex-linked white (eye) locus. Gill (1963) found a mutation which causes males to court both males and females; males may form lines or circles of as many as ten flies, each courting and being courted. Ewing and Manning (1967) reported that no mutants were known at that time which increased mating success and that only one,

forked (a sex-linked recessive bristle mutant), was neutral in its effects. Diederich (1941) considers the differences between wild type and mutant to be due to differences in vigor.

2. Differences Between Strains or Stocks

When different strains and stocks of the same species show differences in mating behavior, it is assumed to be, at least in part, due to genetic differences. With regard to comparisons of inbred and outbred lines, Manning (1963) stated that inbred lines are often characterized by low general and sexual activity. Hoenigsberg and Santibanez (1959) found that outbred melanogaster show homogamic mating preferences and (1960) that inbreeding causes sensory differences which are of consequence in courtship and mating. Koref Santibanez and Waddington reported (1958) that both inbred and isogenic lines showed diminished sexual activity.

Hoenigsberg, Koref Santibanez, and Sironi (1959) found that strains of D. prosaltans and equinoxialis showed significant intrastrain mating preferences, and Kessler (1962) similarly demonstrated that races of D. paulistorum show qualitative and quantitative differences in courtship behavior which affect reproductive isolation. Hildreth and Becker (1962) found that in just three years females of one melanogaster stock (not exposed to artificial selection) had become more receptive to males, presumably due to genetic changes. Parsons (1965) studied five pure (highly inbred) stocks of melanogaster and

found that the stock of origin of the male influences the duration of mating between flies of the different stocks.

The isolation between melanogaster and simulans is greatly affected by the stocks employed. Barker (1962b) tested mutants of the two species and found that isolation varied for various combinations. Although the cross between simulans females and melanogaster males is notorious for its low frequency of occurrence (compared to melanogaster females with simulans males) (Morgan, 1929), the Israel strain of simulans gave the reverse results (Ronen, 1957). Since two different laboratory strains of melanogaster were used, Ronen concluded that the aberrant results must be due to the simulans genome. Barker (1929b) also obtained similar anomalous results but attributed these to the fact that he had paired the more active melanogaster females with the less vigorous simulans males and the less active mutant simulans females with the more active wild type melanogaster males, thereby accounting for the greater success of the latter cross, since the females could not so easily avoid the males. Although Schultz and Dobzhansky (1933) found that wild type simulans males mate less readily with triploid than with diploid melanogaster females of the same triple sex-linked mutant stock (yellow vermilion forked), Pontecorvo (1943) routinely obtained nearly 100% fertile cultures by placing wild type or forked simulans males with triploid melanogaster females.

Spieth and Hsu (1950) found that a Mexico stock of simulans gave almost no intraspecific inseminations (even in

light) compared with a Texas simulans stock. Carmody et al. (1962) found that some strains of D. paulistorum are more sexually excitable and ready to mate than others. In light of the variable results described above, one must agree with Barker (1967) that it is impossible to specify categorically the degree of sexual isolation between two species.

Mating behavior may vary significantly depending on the inversions or gene sequences present. D. persimilis stocks with different chromosome inversions show different mating speeds (Manning, 1967b). Kaul and Parsons (1965) found that mating speed (or time between beginning of courtship and copulation) and duration of copulation (in D. pseudoobscura) are determined by the male karyotype for various combinations of the Standard and Chiricahua gene sequences (St/St, St/Ch, Ch/Ch). Also in pseudoobscura, Parsons and Kaul (1966) found that for various combinations of the Arrowhead and Pike's Peak gene sequences (Ar/Ar, Ar/PP, PP/PP), the female's karyotype was most important in determining mating frequency and the male's in determining speed of copulation. Parsons had earlier (1965) considered that in melanogaster, when mating is rapid, it is possibly determined entirely by the male genotype, with the female reaction becoming more important at a later stage in the courtship.

3. Cytoplasmic Effects

Not all inherited differences may be due to chromosomes, however, since Ehrman (1968) reports that the mating advantage

conferred upon certain male heterokaryotypes varies depending on the source of cytoplasm in the heterozygote. There are other examples of apparent cytoplasmic effects on the mating advantage of male heterozygotes (Petit and Ehrman, 1969). Mayr (1946) considered a possible maternal effect when he found that pseudo-obscura-persimilis hybrid females are inseminated more often when the female parent (of the hybrid) is conspecific with the male being tested with the hybrid.

4. Selection

Selection has often succeeded in altering mating behavior, sometimes as a secondary result. When Ewing (1961) selected for small sized flies (melanogaster), he found that they also were characterized by more wing vibration in the courtship of the males. He demonstrated that this occurred through selection for stimulating elements in the courtship because their small size was less stimulating in courtship. Ewing also found (1963) that flies (melanogaster) selected for different general activity levels showed changed courtship, in that flies with lower activity levels displayed more mating behavior and vice versa. Wallace (1954), using melanogaster, selected for sexual isolation between the mutant strains straw and sepia by culturing them together in cages and removing hybrids (wild type) each generation. After 73 generations there were marked preferences for intra-sepia matings, but straw females were still inseminated equally by both types of

males. Knight (1963) selected against hybridization between ebony and vestigial strains of melanogaster in population cages by removing hybrids (wild phenotype) and obtained increased sexual isolation. Manning (1961) selected successfully for fast and slow mating speed in melanogaster. He tried selecting for fast and slow mating speeds in simulans by selecting only in the males (one-sexed selection) but obtained responses in only the slow-mating lines (1963). MacBean and Parsons (1967) selected successfully for long and short durations of copulations in melanogaster. Robertson, however, reported (1966) that fourteen generations of selection for positive assortative mating (in melanogaster) failed to provide evidence of sexual isolation between two basic populations adapted to different diets (with and without EDTA).

Koopman (1950) started population cages with both D. pseudoobscura and persimilis and obtained a big reduction in hybridization by removing the hybrids each generation (hybrids between these species can mate and have offspring). Kessler (1966) successfully selected for and against sexual isolation between pseudoobscura and persimilis. Henslee (1966) successfully selected, within a parthenogenetic strain of D. mercatorum, for increased sexual isolation from a bisexual strain of that same species. Rendel (1945) selected, in a homozygous witty eye (wi) line of D. subobscura, for females which do accept yellow males as mates. In the F-4, 82.1% of the females accepted yellow males, compared to 7.5% in the controls. (This was a one-sexed selection experiment.)

In accordance with the general success in selecting for sexual isolation and with the characteristics of such selection, its basis is considered to be polygenic. Mather (1941) states that specific differences are always polygenic if the species are biologically isolated. Dobzhansky (1964) considers (for D. pseudoobscura and persimilis) that the female preference for her own males is due to a polygene complex, with genes on all chromosomes. Tan (1946) described the sexual isolation between persimilis and pseudoobscura as due to summation of polygenes on all major chromosomes, and Kessler concluded (1966) that a change in sexual isolation (between these two species) due to artificial selection is an indication of polygenic determination. Patterson (1942) considered the sexual isolation factors for intraspecific crosses in D. repleta to be autosomal recessives and that at least some of the sexual isolation factors for the virilis group are autosomal recessives. King (1965) states that both autosomal and sex-linked loci are involved in sexual isolation in the genus Drosophila. Henslee (1966), however, considered the isolation he obtained (in D. mercatorum) to be due to just a few genes, presumably because of the rapid response.

Other behavioral traits which have been successfully selected for in melanogaster include positive and negative geotaxis (Hirsch, 1962; Dobzhansky and Spassky, 1962), positive phototaxis (Carson, 1958), and different levels of spontaneous activity (Ewing, 1963).

D. Description of Experiments to be Performed

It is apparent from the foregoing that mating behavior in Drosophila is quite labile, depending on the particular environmental and genetic background of the flies involved. I have investigated the sexual isolation between D. melanogaster and D. simulans from both environmental and genetic approaches. A brief description of the major experiments follows.

In the conditioning experiments I studied the effects of the exposure of the two species to each other during early adult life. In particular I wanted to see if melanogaster females which had been counter-conditioned (during their early adult life) with males of the other species (simulans) would display altered sexual isolation with simulans males (during a later period).

In the mixed cultures experiments I studied the effects on sexual isolation of rearing the two species (from egg to adult) in the same, rather than different, culture bottles.

In the selection experiments I attempted to select, in both melanogaster females and simulans males, for increased and decreased sexual isolation from the opposite sex of the other species. Genetic analysis was carried out on the female selection lines in order to determine the relative contributions of the major chromosomes to the selected traits.

CHAPTER II

METHODS AND MATERIALS, GENERAL

There are two ways in which crosses between Drosophila melanogaster and D. simulans can be attempted: melanogaster females with simulans males and simulans females with melanogaster males. It is known that as a rule, the latter cross yields offspring in only a very small percentage of the crosses set up (Sturtevant, 1929), and preliminary work with both types of crosses certainly verifies this. The former cross is the one most often reported successful, and it yielded offspring in a relatively large percentage of the crosses set up in this study.

The offspring produced are different in the two types of crosses. Simulans females mated by melanogaster males yield nearly all male offspring and a few females; melanogaster females mated by simulans males yield nearly all female offspring and a few males. Certain cytoplasm-chromosome incompatibilities are believed to be responsible for this difference.

Except when stated otherwise, all crosses studied were of homozygous yellow melanogaster females with wild type simulans males. Yellow is a sex-linked recessive gene, and the expected offspring from the hybrid cross, wild type females, can be distinguished from progeny resulting from non-virginity, i.e., yellow males and females. The yellow stock was made from a multiple sex-linked marker stock (y ct^6 ras f), and the basic simulans stock was a lab stock maintained for years by mass-culturing.

All crosses were made in eight-dram food vials plugged with cotton and cultured at about 27° C. in a constant temperature incubator in which trays of water maintained high humidity. Constant light was provided by a 40 watt bulb located a few inches above the same shelf on which the vials were placed and between one and three feet from them. A typical twenty-bottle food batch was made from the following recipe: water--one liter, agar--ten grams, methyl parasept--two teaspoons, brewer's yeast--28 grams (in eight ounces water), molasses--four ounces, cornmeal--eight ounces (in 16 ounces water).

All crosses were set up as follows: flies to be crossed were etherized late in the evening (around 9 PM) and distributed among empty vials, one sex per vial; the number of flies per vial varies with the experiment and will be indicated with the explanation for each experiment. At this time, flies were examined for macroscopic abnormalities (shrivelled wing, etc.) because of the importance of various body parts, especially the wings, in courtship, and only normal-appearing flies were used. Three hours later, after the flies had recovered from the effects of etherization, the contents of a "male" and a "female" vial were shaken into a single food vial, which was placed in the incubator. After a certain number of days, to be specified for each experiment, the flies were etherized, the sexes separated, and the flies either discarded or saved for determination of which ones had mated. At this time I also recorded the number of dead flies of each sex, if any, since the

alteration of the sex ratio during the experiment might alter the outcome. Even if some flies in a vial were dead when the time allowed for mating had elapsed, the females were saved for determination of which ones had mated (if this procedure was being employed at the time), and statistical tests were later made to determine whether alteration of the number and sex ratio of flies in a vial influenced the mating results (see Chapter VI, A). I judged whether mating had occurred by the absence or presence of unisexual (female) broods of the appropriate phenotype. Because preliminary crosses showed such different results from one experiment to the next, experimental and control crosses (or crosses involving different lines in a selection experiment) were always set up simultaneously.

CHAPTER III

CONDITIONING

A. Methods and Materials

I wanted to determine whether melanogaster females between three and four days of age hybridize more readily with simulans males if they have been in the company of simulans males for the three preceding days. I made stock bottles of yellow melanogaster, wild type simulans, and yellow simulans. Stock bottles of a particular type were always parented by the same number of flies during any particular experiment, in order to maintain population density relatively constant. However, more simulans were always used to parent simulans bottles in order to compensate for either lower fecundity (DiPasquale and Koref Santibanez, 1960; Tantawy and Soliman, 1967) or the greater loss of simulans pupae which are more often formed on the surface of the medium and suffocated if submerged (Sameoto and Miller, 1968). Female melanogaster collected on day 1 (and therefore between zero and twenty-four hours old) were divided into equal groups: one group was placed, five per vial, in vials each of which contained five yellow simulans males, any offspring by which would be yellow; the other was placed, ten per vial, in vials for storage at room temperature with alternating periods of light and dark, until three to four days of age. In this way all vials, both for storage and mating,

contained ten flies. Since mating behavior may be influenced by the number of stimuli received previously from other flies (Manning, 1959a), it is important to equalize the numbers of flies in the two sets of vials.

At the end of the fifth day, the melanogaster females which were stored without simulans males were placed, five per vial, with five wild type simulans males for four days. These were the control crosses. Also at the end of the fifth day, the melanogaster females which had been together with yellow simulans males on days three through five were removed from those vials (designated the counter-conditioning crosses), etherized, re-examined for abnormalities which might influence mating, and mixed together before being redistributed, five per vial, among empty vials. Each group of five was placed three hours later with five wild type simulans males for four days; any offspring by these males would be wild type. These constituted the experimental crosses. At the end of four days all flies were etherized and, in the first experiment of this sort, discarded. In the second experiment, all surviving females were placed singly in food vials in order to determine exactly what percentage of flies had been inseminated in the controls. In the experimentals, because progeny by the two types of males would be different, placing females singly in food vials allowed for an exact determination of what percentage had been inseminated by the "first" males, the "second" males, and by both.

B. Results

The results of these two experiments are shown in Figure 1 and Table I. (Except when stated otherwise, all statistical tests mentioned in the Results sections were 2 x 2 contingency tables with one degree of freedom, and Yates' correction factor was employed whenever the total sample was less than 40 or the expected number for any class was less than ten. Differences described as significant yielded probability values less than 0.05).

In the first of these experiments only the results of the small mass matings are available, and there is a barely significant difference (P = .02-.05) in the numbers of vials which yield progeny (of wild type simulans males) in the experimental and control crosses. This indicates decreased hybridization after counter-conditioning, as can be seen by comparing columns 1 and 4 on the left side of Figure 1. One should keep in mind, however, that the results of a small mass mating vial may not reflect accurately the mating activities of individual females.

There is no significant difference between the numbers of vials which yield progeny in the pre-experimental (counter-conditioning) crosses and those which yield progeny from the "first" males in the experimental crosses (columns 2 and 3, respectively, on the left side of Figure 1). This may indicate (1) that the presence of yellow female progeny in the experi-

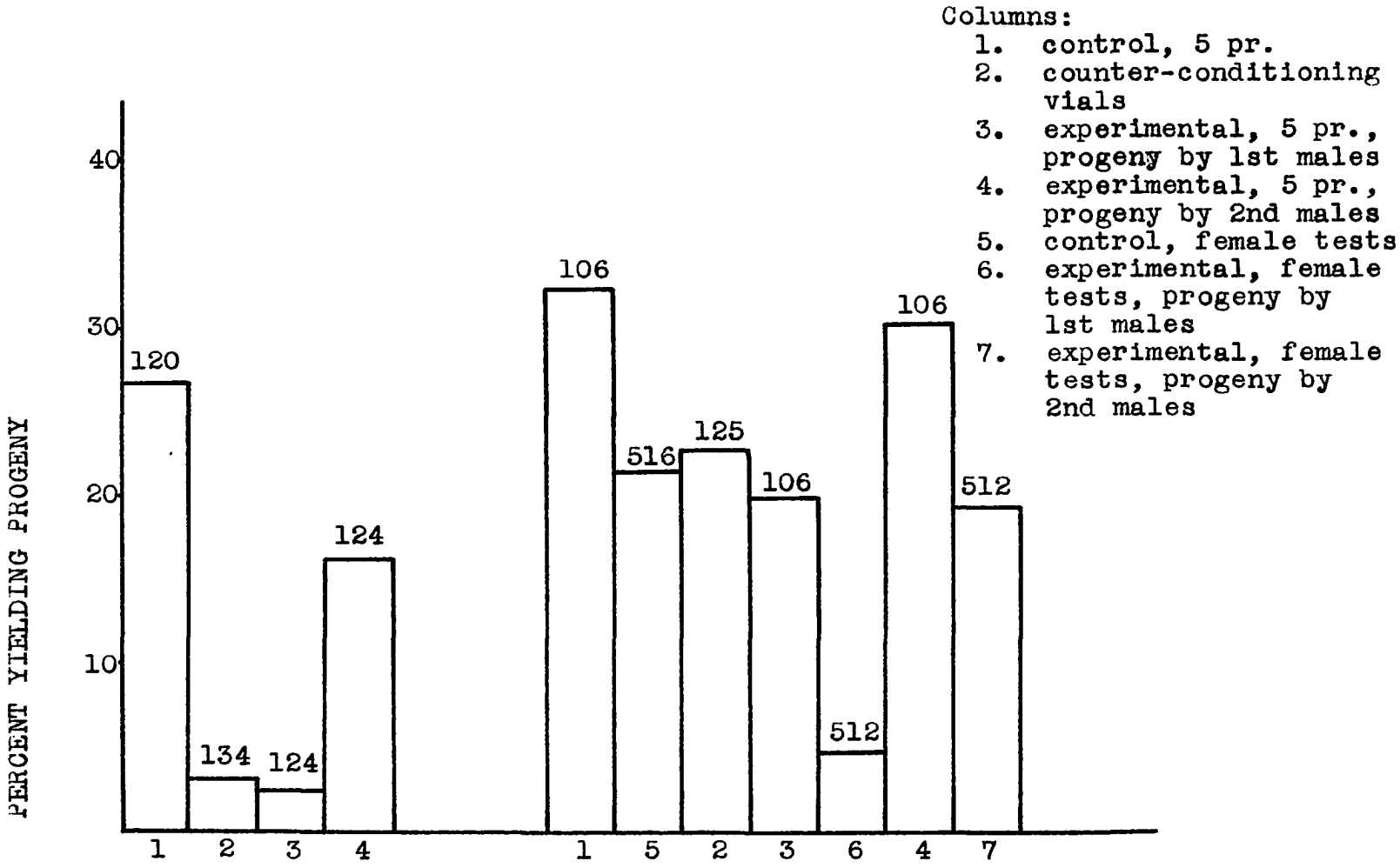


Figure 1. Counter-conditioning. Percent of vials yielding progeny. Numbers above columns are sample sizes. First experiment is on left, second experiment on right.

Table I

Results of Counter-conditioning

Type of cross	no. of 5 pr. matings	number yielding progeny	%	no. of females tested	no. of females inseminated	%
Experiment 1 (3-1969)						
control	120	32	26.7	0	--	-
experimental	124	3(by 1st) 20(by 2nd) 0(both)	2.4 16.1 0.0	0	--	-
counter-conditioning	134	4	3.0	-	--	-
Experiment 2 (9-1969)						
control	106	34	32.1	516	110	21.3
experimental	106	21(by 1st) 32(by 2nd) 6(both)	19.8 30.2 5.7	512	24(by 1st) 98(by 2nd) 1(both)	4.7 19.1 .2
counter-conditioning	125	28	22.4	-	--	-

mental vials is indeed an accurate measure of whether mating occurred in the counter-conditioning vials, since the same females are present in both cases but simply redistributed, and that subsequent etherization and handling did not preclude the laying of fertilized eggs; and (2) that virgin and uninseminated females from the counter-conditioning vials were indeed distributed at random among the experimental vials (after apparent random mating in the counter-conditioning vials).

In the second experiment individual females were scored for inseminations. For both the five-pair matings and the female tests there is no significant difference between the numbers yielding progeny by wild type males in the control and experimental crosses (columns 1 vs. 4 and 5 vs. 7, respectively, on the right side of Figure 1). Nor is there any difference between the numbers of five-pair mating vials yielding progeny of "first" males in the counter-conditioning and experimental crosses (columns 2 and 3, respectively, on the right side of Figure 1). This would again seem to verify that the presence of progeny of "first" males in the experimental vials is an accurate measure of whether females were mated by "first" males in the counter-conditioning vials, and that virgin and inseminated females from the counter-conditioning were distributed at random among the experimental vials.

In the female tests, there is only one female which yielded progeny of both "first" and "second" males, compared to the nearly five expected if the two types of inseminations were independent events. A test for independence indicates

that an insemination by a "first" male significantly reduces, though barely so, the chances of an insemination by a "second" male (see Appendix, 1).

Two things should be noted about the experiments as a whole. One is the difference between the first and second experiments in frequency of hybridization, especially in the counter-conditioning vials (3% and 22.4%, respectively, in columns 2 of Figure 1). The two experiments were run, however, at very different times of the year (March and September, respectively). The other point is the large difference between the percentage of females inseminated and the percentage of small mass mating vials which yield offspring (Table I). To not determine exactly what percentage of the females is inseminated results in an overestimate of hybridization, since the insemination of just one female can cause a five-pair mating vial to be scored as a mating.

C. Discussion

Counter-conditioning the young (one to two day) melanogaster females with simulans males had no effect on later isolation, judging from the experiment in which individual females were tested singly. This is not in agreement with the findings of Pontecorvo (1942), who found that once a young melanogaster female mates with a simulans male, successive matings occur till old age, as though the mating reaction of the young female is not yet fully determined but still liable to conditioning by

foreign males. The results of my first experiment, which showed significantly increased isolation (after counter-conditioning) for the five-pair mating vials, is difficult to interpret since the individual females were not tested for insemination, and not enough of these experiments were performed to determine how the time of year might influence these types of crosses (especially since the two experiments were performed in very different times of the year, March and September, respectively, for the first and second experiments). When Mayr and Dobzhansky (1945) counter-conditioned D. pseudoobscura males with persimilis females, the males later showed greater isolation from persimilis (under poorly controlled conditions); persimilis males counter-conditioned with pseudoobscura females later showed significantly decreased isolation with pseudoobscura. It should be noted that Mayr and Dobzhansky studied counter-conditioning of males, whereas Pontecorvo and I studied counter-conditioning of females.

CHAPTER IV

MIXED CULTURES

A. Methods and Materials

To investigate possible effects of the larval and pupal environment on hybridization, I reared the two species (*melanogaster* yellow, *simulans* wild type) together and separately. These species have four stages in their life cycle: egg, larva, pupa, and adult; usually the egg, larval, pupal, and early adult stages are passed in monospecific cultures, with members of only the same species nearby. In preparation for rearing the two species together in mixed cultures, *simulans* males and females were left in bottles for 24 hours and followed by *melanogaster* males and females. This sequence was adopted because Moore found (1952) that *simulans* females lay almost no eggs where *melanogaster* has laid eggs, and a few preliminary trials confirmed that this is so. The two species have nearly identical rates of development at the temperatures employed (Moore, 1952), but *simulans* seemed to eclose slightly earlier in these experiments. Since the experimental design involved using *simulans* males which were one day older than *melanogaster* females, it would have been of some advantage if the faster-developing *simulans* parents followed the *melanogaster* parents in the bottles. In the method used, however, the earliest eclosing *simulans* males were "wasted" since there were no *melanogaster* females eclosing in numbers until nearly two days later.

For any particular experiment the numbers of flies used to parent the mixed cultures were constant. Control cultures were parented by numbers of simulans or melanogaster males and females expected to yield the same number of progeny per bottle as in the experimental mixed cultures. Seven major experiments were run. In the first six, both experimental and control cultures were cleared of flies every two hours in order to reduce the possible influence of early contact with the same or the other species on mating behavior (pro- and counter-conditioning, respectively). The seventh such experiment was designed differently and will be described in more detail separately. In all experiments the same time schedule was followed as for the previous experiment, with four to five day old simulans males placed with three to four day old melanogaster females for four days (five pairs per vial). In the first five experiments all flies were discarded after four days so that only the mass mating vials could be scored for the presence of offspring. In the last two experiments, however, males only were discarded after four days, and the females were placed singly in vials, in order to determine exactly what percentage of females had been inseminated. A mass mating vial which is scored as a "mating" could yield either one, two, three, four, or five inseminated females, so that the results obtained by the two methods could be quite different. In the sixth experiment there were three environmental treatments instead of two. Control crosses were set up using flies from

monospecific cultures, and experimental crosses were set up in two ways: (1) mating flies from the same mixed culture bottle, (2) mating flies from any mixed cultures, as was done in the first five experiments. These last two crosses are referred to as "intra-bottle" and "mixed", respectively. In the seventh experiment, flies were cleared only every six hours, to insure virginity in the females, and there were three environmental treatments. Not only were control cultures parented by one species only and mixed cultures by both species, but some mixed cultures were parented by flies of both species which had passed their egg, larval, pupal, and very early adult lives in mixed cultures. The purpose of this was to see if the effects on hybridization, if any, of being reared together are limited to that particular generation or are strengthened by an additional generation of being reared together.

Three subsidiary experiments in the mixed cultures study were run (with relatively small numbers of flies) just to make sure that changing the age of the flies or the length of time they are together do not cause striking differences in the outcome. In all three experiments, only flies from the same bottle were placed together, in the experimentals; controls were as before. In the first such experiment, flies of the same age as before were used but were left together only two days in order to investigate whether the differences in frequency of hybridization between the two types of flies exists after two days. In the second and third subsidiary experiments,

females of only one to two days of age were used and placed with simulans males for either two or four days. For all three experiments females were placed singly in vials in order to determine the percentage inseminated.

B. Results

The results of the first five experiments are shown in Figure 2 and Table II. Although in each case but the last, which was a very small sample, there is an indication of decreased isolation among flies eclosing in mixed cultures, this difference was significant for only the fourth experiment. By combining the probabilities from the independent tests of significance (except for the fifth experiment, which had a sample size only slightly larger than one-third the size of the next largest experiment) (Sokal and Rohlf, 1969), a significant reduction in isolation was found after mixed culturing. There is considerable variation in success of hybridization from one experiment to the next, with experiments in winter months yielding more matings.

The results of the sixth experiment are shown in Figure 3 and Table II. There is an increase in frequency of hybridization from control crosses, made with flies from separate cultures, to "mixed" experimental crosses, made with flies from any mixed cultures, to "intra-bottle" experimental crosses, made with flies from only the same mixed culture. The only significant difference between five-pair matings is between the

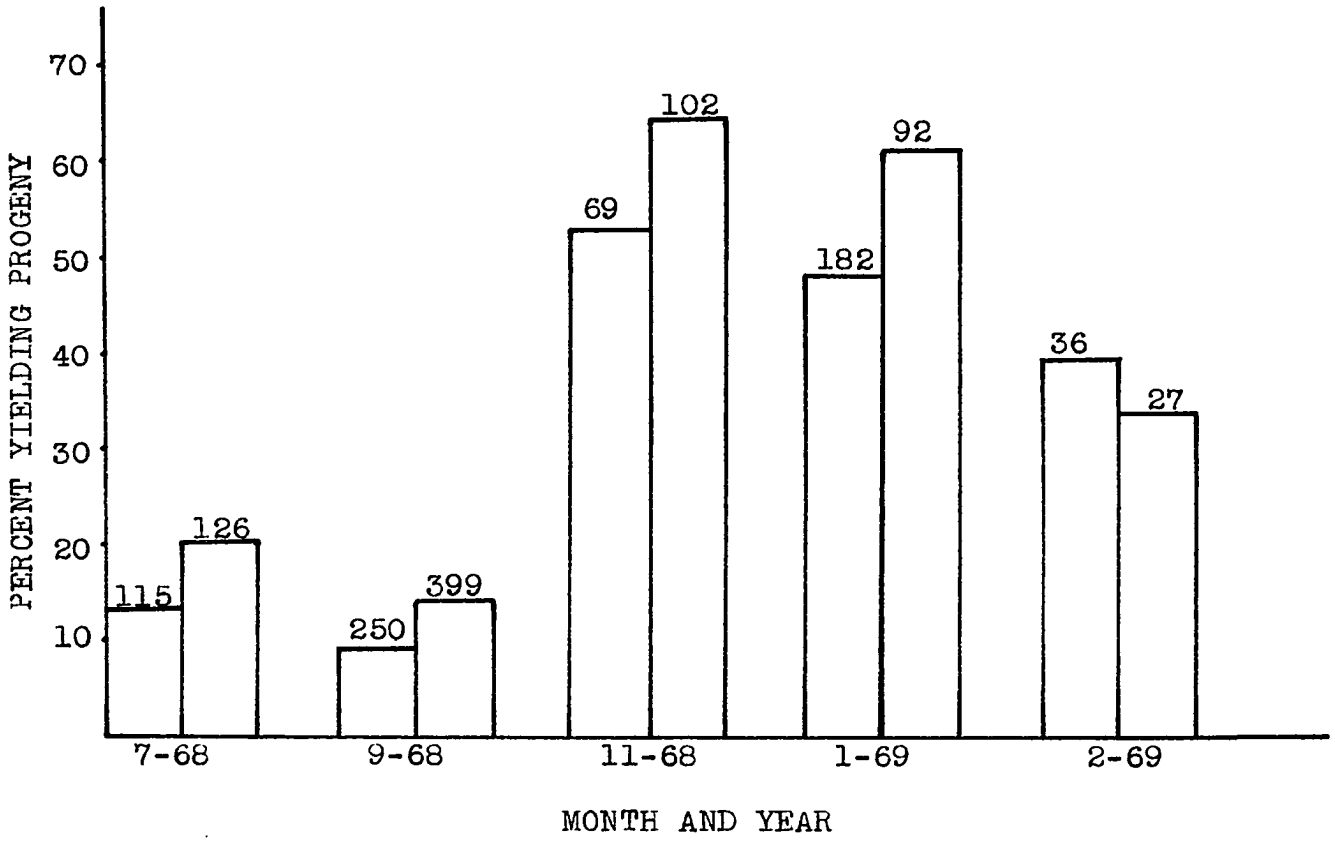


Figure 2. Mixed cultures, experiments 1-5. Percent of five-pair mating vials which yield progeny. Control is on left in each case. Numbers above columns are sample sizes.

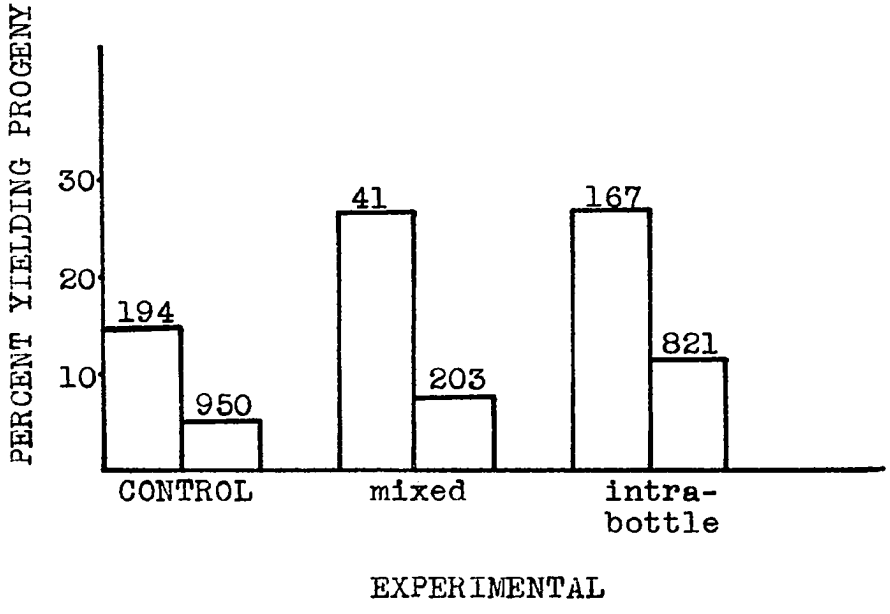


Figure 3. Mixed cultures experiment no. 6. Percent of five-pair vials and single females which yield progeny. Five-pair vials are on the left in each case. Numbers above columns are sample sizes.

Table II

Results from Mixed Cultures Experiments 1-6

Experiment		no. of 5 pr. matings	% with offspring	no. of females tested	no. of females inseminated	%
1.	control	115	13.04	0	-	-
	mixed culture	250	20.00	0	-	-
2.	control	126	8.73	0	-	-
	mixed culture	399	13.78	0	-	-
3.	control	69	52.17	0	-	-
	mixed culture	102	63.72	0	-	-
4.	control	182	47.80	0	-	-
	mixed culture	92	60.87	0	-	-
5.	control	36	38.89	0	-	-
	mixed culture	27	33.33	0	-	-
6.	control	194	14.95	950	51	5.37
	mixed	41	26.83	203	16	7.88
	intra-bottle	167	26.95	821	97	11.81

controls and the intra-bottle experimentals (columns 1 and 5, respectively, in Figure 3). Significant differences between numbers of individual females inseminated existed for both control vs. intra-bottle and mixed vs. intra-bottle comparisons (columns 2 vs. 6 and 4 vs. 6, respectively, Figure 3).

The results of experiment seven are shown in Figure 4 and Table III. The original results were anomalous when compared with the above experiments in that there was a significantly increased isolation (in the single female tests) among flies from one generation of mixed culturing (which is comparable to the above experiments) when compared with the controls. Re-examination of the data, however, revealed that it was the almost complete isolation between flies reared in one bottle that caused the anomalous results. After subtracting these data from the rest, there was no significant difference between controls and flies reared in mixed cultures for one generation. Both the original and modified results, as well as those of the aberrant bottle, appear in Table III, but only the modified results are shown in Figure 4. By contrast, a comparison of the results from the two bottles of two-generation mixed cultures revealed no significant differences. Flies from two generations of mixed culturing showed significantly decreased isolation when compared with both the controls and the one-generation mixed cultures. (The controls and experimentals were run simultaneously; see Methods and Materials, General). When compared with the controls, there were significant differences for both the five-pair mating vials and the single

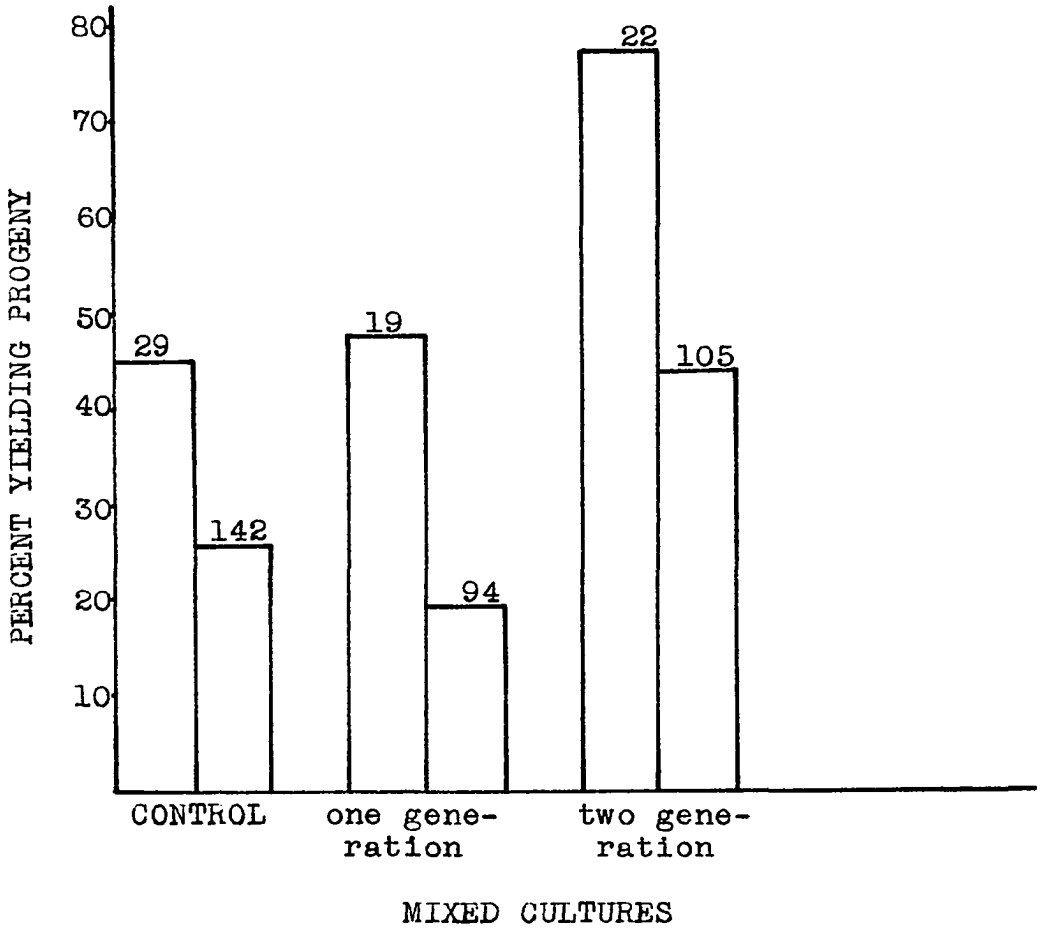


Figure 4. Mixed cultures experiment no. 7. Percent of five-pair vials and single females which yield progeny. Five-pair vials are on the left in each case. Numbers above columns are sample sizes.

Table III

Results from Mixed Cultures Experiment 7
(5-1970)

Experiment	no. of 5 pr. matings	% with offspring	no. of females tested	no. of females inseminated	%
Original results					
control	29	44.83	142	36	25.35
one gen. mixed culture	26	42.31	128	19	14.84
two gen. mixed culture	22	77.27	105	46	43.81
Results from aberrant bottle					
one gen. mixed culture	7	14.29	34	1	2.94
Modified results					
one gen. mixed culture	19	47.37	94	18	19.15

female tests (columns 5 vs. 1 and 6 vs. 2, respectively, in Figure 4). When compared with the one-generation mixed cultures, the two-generation mixed cultures showed significant differences only in the single female tests (columns 6 and 4, Figure 4). Since the sample sizes in this set of experiments are considerably smaller than in the sixth experiment, in which reduced isolation due to mixed culturing was clearly shown, it is possible that the absence of reduced isolation in the one-generation mixed culture crosses is due to sampling error, especially since all of the flies for the mixed culture experiments came from only four bottles (two for the one-generation mixed cultures, two for the two-generation mixed cultures) and the presence of an unusual environment in just one bottle might affect the results. There were no observable differences between the bottles, but the use of a smaller number of flies from the aberrant bottle may indicate a smaller population. It's also possible that differences in the microbial population in the aberrant bottle caused differences in chemical products, odors, etc. This experiment differs from the first six, as described earlier, in that flies were cleared only every six hours instead of every two hours, but it seems unlikely that increased contact with flies of the same and the other species would result in different degrees of isolation in the case of one generation of mixed culturing and two generations of mixed culturing.

The results of the three subsidiary experiments are shown in Figure 5 and Table IV. When the experimental design

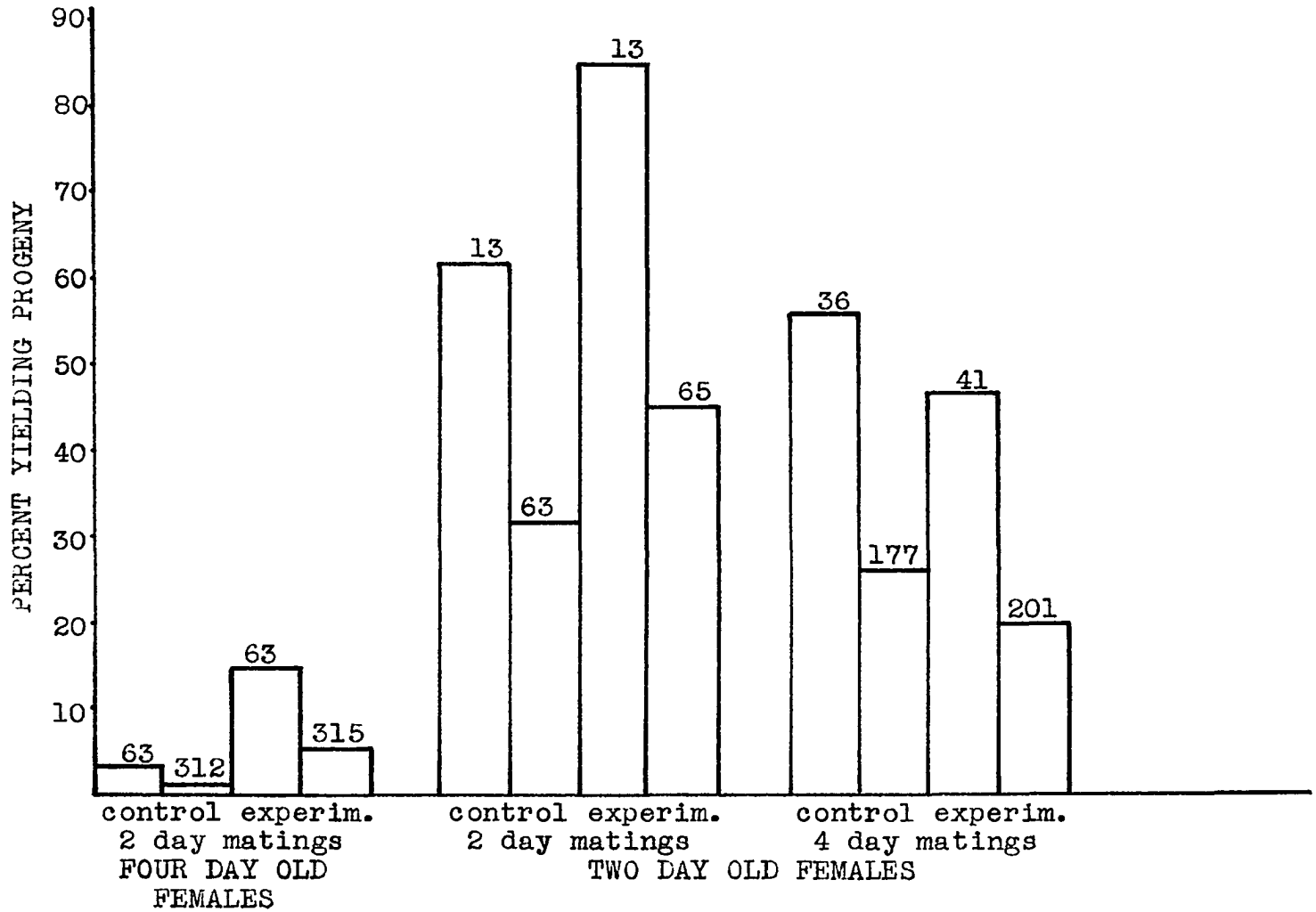


Figure 5. Subsidiary mixed culture experiments. Percent of five-pair vials and single females which yield progeny. Five-pair vials are on the left in each case. Numbers above columns are sample sizes.

Table IV

Results from Subsidiary Mixed Cultures Experiments
(August 1969)

Experiment	no. of 5 pr. matings	% with offspring	no. of females tested	females inseminated	%
two day matings with old (3 to 4 day females)					
control	63	3.17	312	3	.96
mixed culture	63	14.28	315	15	4.76
two day matings with young (1 to 2 day) females					
control	13	61.54	63	20	31.75
mixed culture	13	84.62	65	29	44.62
four day matings with young (1 to 2 day) females					
control	36	55.56	177	46	25.99
mixed culture	41	46.34	201	39	19.40

was exactly as in the sixth experiment, using experimental flies from only the same mixed culture (intra-bottle experimental), except with only two days in which to mate instead of four, there is again a significantly reduced isolation in the experimental crosses (for the single female tests, columns 4 vs. 2, Figure 5). When the only changes in experimental design are to employ one to two day old females (instead of three to four day old females), there is no significant difference between controls and experimentals, regardless of whether the flies are left together for two or four days. However, the sample sizes using younger females were small and may not provide random sampling or statistical significance for the particular magnitude of difference observed. It can be noted, however, that in experiments with young females, a much greater percentage of females is inseminated. It should be kept in mind that the purpose of these subsidiary experiments was solely to determine whether these changes in experimental design would produce striking differences in results compared to the other experiments.

C. Discussion

The various mixed cultures experiments showed some consistency. Especially when female tests are performed and sample sizes are large, there is significantly decreased isolation between melanogaster females and simulans males which come from the same culture. There is an indication, from less

extensive experiments, that the decreased isolation can be detected after the males and females are together for only two days (rather than four) and that the effect of mixed culturing may be enhanced with an additional generation of mixed culturing. The former result may indicate that the difference in mating frequency in the experimental and control crosses is found throughout the four day mating interval. This effect, however, as well as that of additional generations of mixed culturing, would have to be verified by replicate and more extensive experiments before definite conclusions are drawn.

Although Manning reported (1959b) that species-specific scents are relatively independent of the environment in their development and that all attempts to influence sexual isolation between these two species by rearing them together have failed, he provided no data or description of techniques used. His findings may very well not be incompatible with the results of this study, when one considers the alterations in experimental design required to demonstrate the effect in these experiments. He did state, however, that such an effect would be more difficult to show for melanogaster and simulans because their normal isolation is so much greater than for pseudoobscura and persimilis, between which the isolation was generally increased by mixed culturing, although with some variation from one experiment to the next (Mayr and Dobzhansky, 1945). Rendel (1945) did not succeed in altering the isolation between wild type females and yellow males (of D. subobscura) by rearing them in the same bottle.

It would seem difficult to attribute this effect of mixed culturing to other than chemosensory mechanisms, and the evidence from the literature certainly emphasizes the importance of the chemical sense in sexual discrimination and other activities as well. For example, Barrows (1907) reported that melanogaster finds food by smell, and that when this scent is lost, as by removal of antennae, the flies find food by accident; Begg and Hogben (1943) confirm that the antennae are the chief olfactory organs involved in the search for food. According to Jacobson (1965), females of several species of Diptera lure the males and, in D. victoriana, males produce an aphrodisiac (or attractant) for the females; victoriana virgins without antennae fail to accept their males due to the absence of olfactory stimuli.

With regard to the role of the chemical sense in sexual isolation, Manning, who has done much of the work on the mating behavior of these two species and their isolation, has discussed this rather extensively (1959a,b; 1965; 1967), and the following discussion of the role of the various senses in sexual isolation is from his papers, except when stated otherwise. That chemoreception may be involved in sexual isolation is morphologically possible since the Diptera are well provided with contact chemosensory hairs on the labellum, tarsi, ovipositor, and wings, and female discrimination (in melanogaster and simulans) is almost certainly based on scent and other chemical differences, for they reject foreign males from the first of

courtship before they can sample their behavior. Discrimination is little affected, however, by the removal of the antennae, which are the main organs for reception of air borne chemical stimuli (Ewing and Manning, 1963). This chemical reinforcement of sexual isolation between melanogaster and simulans and the increased discrimination with age are due to a strengthened species-specific scent which is largely concentrated on the body surface, although some is probably air borne, and it is largely by contact chemoreceptors, then, that females distinguish their own from the other males. The males may also discover from tapping if the female is of his own species, and presumably a specific chemical in the wax of the epicuticle matches up with the male chemoreceptors. Chemical changes affecting scent have been of major importance, then, in the development of isolating mechanisms in the genus Drosophila.

Although other means for isolation between melanogaster and simulans have been proposed, female discrimination involves neither visual stimuli, since the two species are nearly identical (at least to human eyes--my qualification), nor courtship sampling. There is no firm evidence that females identify the males by courtship behavior, which is not a good basis for discrimination because variations in such behavior are quantitative and not clear cut and distinct enough (at least to human observers--my qualification). Although Bennet-Clark and Ewing (1970) consider the fruit fly "love song" a powerful isolating mechanism, this does not explain the usual breaking off of

interspecific courtship after the male taps the female (which is not confined to melanogaster and simulans but is also found in the D. paulistorum complex, Kessler, 1962), and there is no advantage to a discriminating mechanism which requires the female to sample several minutes of the male's courtship to determine whether he is foreign, although the courtship displays may help to emphasize scent distinctions through the wafting of odors by wing vibrations, for example. Chemical differences are likely to arise early in divergent populations, and in the two cases in which behavioral data on the effects of selection for increased isolation are available (Koopman, 1950; Pearce, 1962), discrimination, not courtship, is altered. The rareness of hybrids in the subgenus Sophophora indicates that sexual isolation is usually based on the female's ability to identify scents and less on sampling the courtship display. Although some have thought that courtship differences provided a means for discrimination on crowded food sources, other Diptera (Tachinidae and Anthomyiidae) which gather on the same food source have no courtship, and yet their sexual isolating mechanisms, apparently involving discrimination by contact, are as effective as those in Drosophila. Similarly, in some spiders the stimulus of touch in species recognition (in courtship behavior) comes from a substance on the female cuticle (Dobzhansky, 1964).

Chemical influences of the larval environment similar to those postulated for the mixed cultures experiments have been called olfactory conditioning by Cushing (1941), who found

that strains of D. guttifera raised in different media (with and without fungus) showed definite preferences in egg laying for the type of medium in which they were raised. Thorpe (1939) found similar decreases in aversion to essence of peppermint (in melanogaster) after larvae were reared in peppermint scented medium and called this pre-imaginal conditioning, because adults from larvae or pupae which were washed free of all peppermint scented medium before pupation or eclosion still displayed a preference for essence of peppermint in choice situations. Arnold and Moray (1964) confirmed that flies reared with essence of peppermint show a strong preference for it in a choice situation. When Moray and Connolly (1963) selected for increased aversion to peppermint in flies reared with it, the aversion decreased (for which they offered no explanation). When they relaxed selection in the F-3 and F-7, aversion rose to normal after relaxation in the F-3, but not after relaxation in the F-7. The short-term effect (or rise in aversion to normal) they called habituation and considered it to be probably a within one generation effect. Hershberger and Smith (1967) criticize Thorpe for calling his results olfactory conditioning and for saying that it was due to habituation. They point out that habituation supposes that scent exposure alone is enough to condition an insect, and that learning (the explanation they prefer) supposes a conditioning which depends on an association of scent with a reinforcing stimulus (food). They then describe experiments of their own, involving various combinations of

peppermint scent with and without food, which demonstrate that the preference for peppermint is a form of learning (since peppermint scent presented without food extinguished the acceptability of the scent). In light of the above discussions, the possible causes of reduced isolation after mixed culturing are as follows: (1) reduced differences in species-specific scents due to a common food source, similar microbial fauna, etc.; (2) habituation, in which scent exposure alone is enough to condition the insects to behave differently (resulting in decreased rejection of the other species); (3) learning, in which the flies associate scents of the other species (or scents held in common due to mixed culturing) with food, and discrimination is reduced. Since none of the experiments performed involved a separation of the scents from the food source, it will be difficult, if not impossible, at this time to determine the exact cause(s) of reduced isolation.

There is an indication that successive generations of mixed culturing cause a greater decrease in isolation between melanogaster and simulans, although repetition of such work will be needed for confirmation. Clutterbuck and Beardmore (1961) studied the numbers of flies which chose food adulterated with various substances and the numbers of eggs laid on such media. They found that the greatest rise in percentage of flies choosing an adulterated food and in percentage of eggs laid on the adulterated food occurred after one generation of being cultured with the substance (for both peppermint and juniper oil) and

that the percentage rose lightly more after six generations of conditioning. Some adulterants (lavender oil) caused increasingly reduced preferences by the insects after more generations of conditioning. In light of their findings that some mutants are repelled by lavender oil while others show no change in response to food scented with lavender oil, it is possible that the reduced isolation between melanogaster and simulans will vary with the stocks employed.

CHAPTER V

SELECTION

A. Female Selection

1. Selection

a. Methods and Materials. I selected melanogaster females for increased and decreased isolation from simulans males. The procedure was as follows: in the initial population, three to four day old yellow melanogaster females were placed with four to five day old wild type simulans males for four days, at the end of which time the males were discarded and the females placed singly in vials in order to determine exactly which ones had mated. Those which had done so were mated with yellow stock male (melanogaster) sibs in order to parent the F-1 generation of the increased hybridization or "up" line, and those which had not accepted simulans males were likewise mated with yellow stock male sibs in order to parent the F-1 generation of the decreased hybridization or "down" line. This procedure was repeated each generation except for one, when selection was relaxed due to the relatively small numbers of offspring. The simulans males were always from unselected stocks. In addition, one and two day as well as four day matings were set up during the later generations in order to select (in the up line) females which had mated with simulans males after just 24 or 48 hours, while still selecting (in the down

line) females which had not mated after even 96 hours, thereby hopefully increasing the intensity of selection in the "up" line. One and two day matings were always set up in both lines, however, in order to provide additional comparisons between the lines, since I considered it possible that differences between the lines resulting from selection might show up in matings of one duration and not those of another. In other words, in each generation beginning with the F-17, all of the females eclosing in each line were divided into three equal-sized groups; one group was used for one day matings, one group for two day matings, and one group for four day matings. I then chose, as parents of the next generation in the up line, as many females as possible which had mated after just twenty-four hours, but in order to have enough females to parent the next generation, females which had mated within 48 hours were often also used, and, if need be, females which had mated, but within 96 hours, were employed also. The parents, then, of the flies used for one, two, and four day matings were the same each generation. Although the number of female parents chosen varied from generation to generation (but was seldom less than ten), it was the same in both up and down lines beginning with the F-5 in series 1 (see below) and the F-1 in series 2. In this way, I attempted to maintain similar population densities in cultures of the two lines.

Beginning with the F-5 of series 1 (see below) and the F-1 of series 2, the selected females were always mated with

their male sibs (rather than with males from the unselected stocks) in order to parent the next generation. Since even females which were selected to parent the next generation had to mate with males of their own species in order to contribute flies to the next generation, there was also a degree of selection for intraspecific mating in both lines.

This female selection experiment was carried out using two stocks. One was the yellow stock made as described earlier from a multiple marker stock (series one female selection). The other (series two female selection) was synthesized from six yellow melanogaster stocks: the series one female selection stock and stocks received from Oak Ridge, Berkeley, Washington University, Pasadena, and East Lansing. Ten pairs of flies from each stock were allowed to mate in separate vials and then to mate and lay eggs together in bottles. Offspring selected at random after a large number of F-1 had emerged were used to parent the series two yellow melanogaster stock, which now presumably contained considerable genetic variability on which to exert artificial selection.

b. Results. The results of series one are seen in Figures 6 and 7 and Table V. In Figure 6 the response to 21 generations of selection (selection was relaxed in the F-5 due to the eclosion of only a small number of flies) is clearly seen for the four day matings. Although there were no significant differences between the up and down lines until the F-9, significant differences (all of them in the direction expected

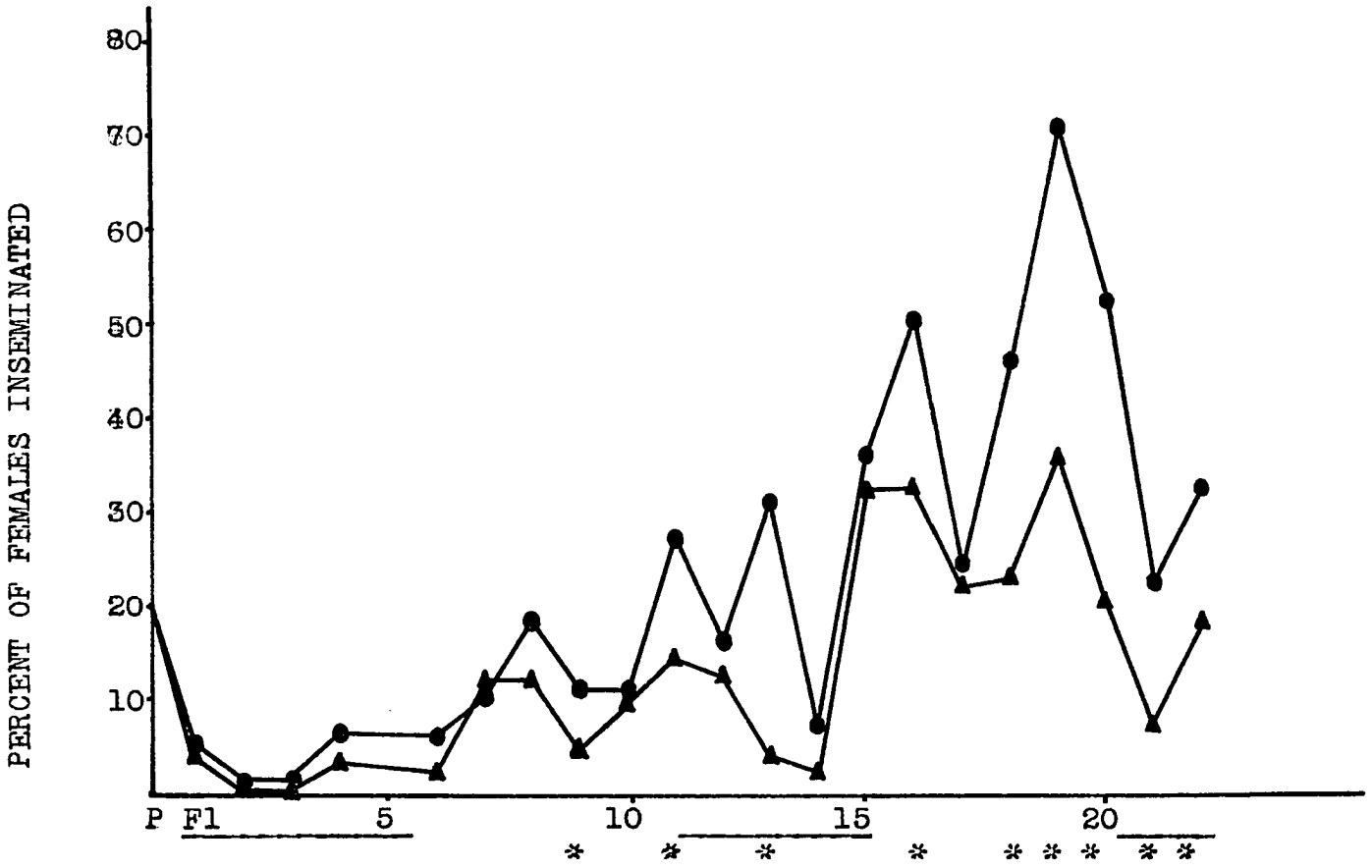


Figure 6. Female selection series 1, four day matings. Percent of females inseminated each generation. Underlined generations are in the April-Sept. interval. Starred generations show significant differences in the expected direction. ● = up line, ▲ = down line.

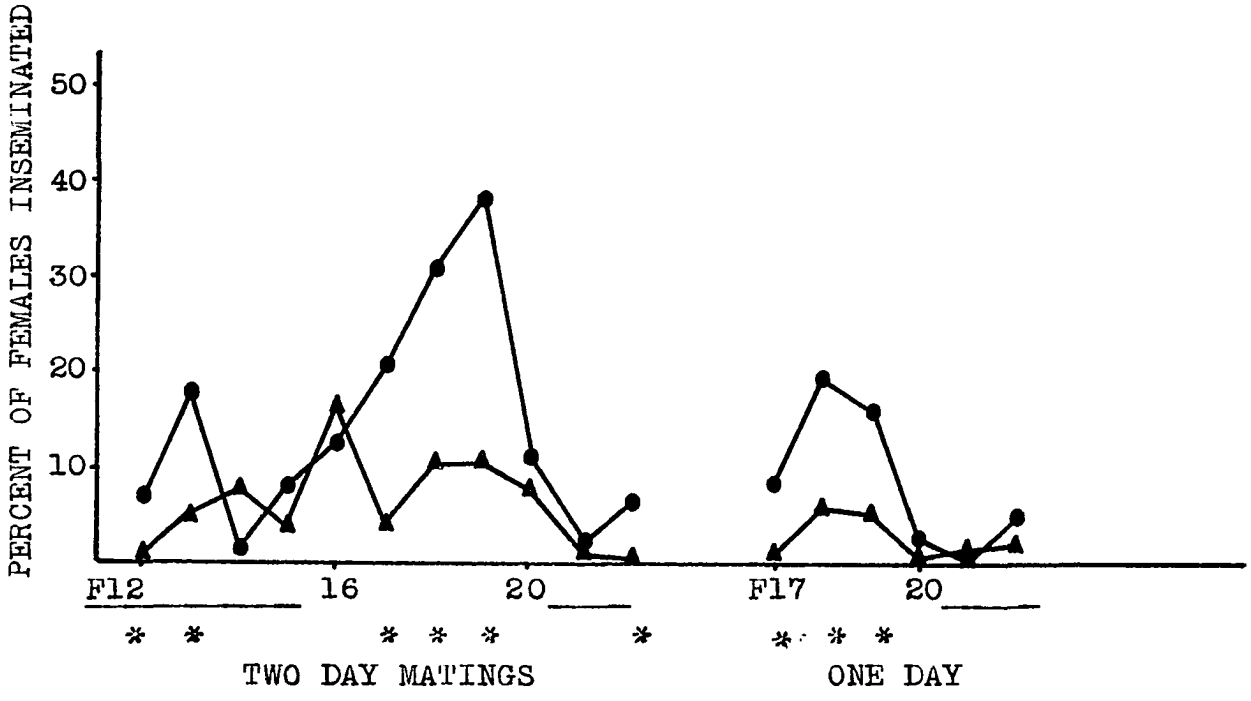


Figure 7. Female selection series 1, two and one day matings. Percent of females inseminated each generation. Underlined generations are in the April-Sept. interval. Starred generations show significant differences in the expected direction. ● = up line, ▲ = down line.

Table V

Results of Female Selection Series 1

Generation	dates	UP no. of 5 pr. vials	UP no. of female tests	UP % in-semi-nated	DOWN no. of 5 pr. vials	DOWN no. of female tests	DOWN % in-semi-nated
P	3-1968	24	107	19.63	24	107	19.63
F-1	4-1968	25	119	5.04	25	121	4.13
F-2	5-1968	22	107	0.93	35	164	0.61
F-3	6-1968	20	97	1.03	20	92	0.00
F-4	7-1968	34	184	6.52	52	249	3.21
F-5		no selection			no selection		
F-6	10-1968	43	207	6.28	34	164	2.44
F-7	11-1968	41	178	10.11	41	177	11.86
F-8	12-1968	44	203	18.23	34	159	11.95
F-9	1-1969	34	159	11.32	34	157	4.46
F-10	2-1969	35	171	11.11	37	181	10.50
F-11	3-1969	41	192	27.08	40	186	14.52
F-12 2-day	4-1969	31	154	5.84	31	152	.66
4-day		30	147	16.33	31	154	12.99
F-13 2-day	6-1969	25	122	17.21	25	122	4.92
4-day		25	123	30.89	25	123	4.06
F-14 2-day	7-1969	21	105	.95	21	105	7.62
4-day		21	101	6.93	21	101	1.98
F-15 2-day	9-1969	17	82	7.32	16	80	3.75
4-day		17	83	36.14	17	83	32.53
F-16 2-day	10-1969	15	74	12.16	15	75	16.00
4-day		14	70	50.00	14	70	32.86
F-17 1-day	11-1969	23	109	7.34	23	113	.88
2-day		23	114	20.18	23	115	3.48
4-day		23	111	24.32	23	111	22.52
F-18 1-day	1-1970	24	118	18.64	24	117	5.98
2-day		24	120	30.00	23	111	10.81
4-day		24	118	46.61	24	117	23.08
F-19 1-day	2-1970	22	108	15.74	22	108	5.56
2-day		22	103	37.86	22	109	11.01
4-day		22	107	71.03	22	108	36.11
F-20 1-day	3-1970	22	110	2.73	22	108	1.82
2-day		22	108	11.11	22	108	7.41
4-day		21	101	52.48	21	102	20.59
F-21 1-day	4-1970	19	93	0.00	19	95	1.05
2-day		22	109	2.75	21	105	1.90
4-day		25	124	22.58	25	125	7.20
F-22 1-day	6-1970	23	113	4.42	23	115	2.61
2-day		23	114	6.14	23	114	0.00
4-day		23	110	32.73	23	113	18.58

if selection is effective) followed in the F-11, 13, 16, 18, 19, 20, 21, and 22. There is also an overall increase in frequency of hybridization in both lines during the course of the experiment. It should be recalled that there was a degree of selection, in both up and down lines, for females which accepted their male sibs as mates, since it was only these females which contributed offspring to the next generation. It is possible that this selection for intraspecific mating has increased the receptivity (to any males) of females of both lines.

The intensity of selection was not increased in the up line until the F-12 (with two day matings) and the F-17 (with one day matings). The response to selection for the one and two day matings is shown in Figure 7. For the two day matings there are significant differences (all of them in the expected direction) in six of the eleven generations (F-12, 13, 17, 18, 19, 22), and in only two of the eleven generations are up line percentages (inseminated) less than those in the down line, and in both cases the samples were among the smallest in the entire experiment (see Table V, F-14 and 16, two day matings). For the one day matings there are significant differences (all of them in the expected direction) in three of the six generations (F-17, 18, 19), and in only one of the six generations is an up line percentage (inseminated) less than that of the down line, and in this case the sample of one day matings was the smallest in the entire experiment (see Table V, F-21, one day matings). The influence of the time interval available for

mating upon the success of the crosses is clearly seen in Figure 8, where the one, two, and four day matings are compared for their percentages of females inseminated.

By combining the probabilities from the independent tests of significance, it was found that although generations one through twelve showed no significant differences between the two lines, generations thirteen through twenty-two did show a significant difference (in the expected direction).

After transforming the differences between the two lines (for each generation) to arcsines, I performed a regression analysis upon the data. The results indicated that a large and significant portion of the variance of the difference between the two lines was explained by regression on number of generations of selection (see Appendix, 2).

After the selection experiment had run for about a year, it became apparent that there was a reduced frequency of hybridization during the summer months. At the end of the experiment after nearly three years of selection, involving parts of three summers and three winters, the seasonal effect, as it will be called, is clearly seen. In Figures 6 and 7, as well as in all figures having to do with the selection experiments, those generations which are underlined on the horizontal axis are those which fell in April through September. Most of the generations with extreme reduction in mating frequency fall during this interval, and most of the peaks fall outside it. When all the results of the two lines (percentages of females

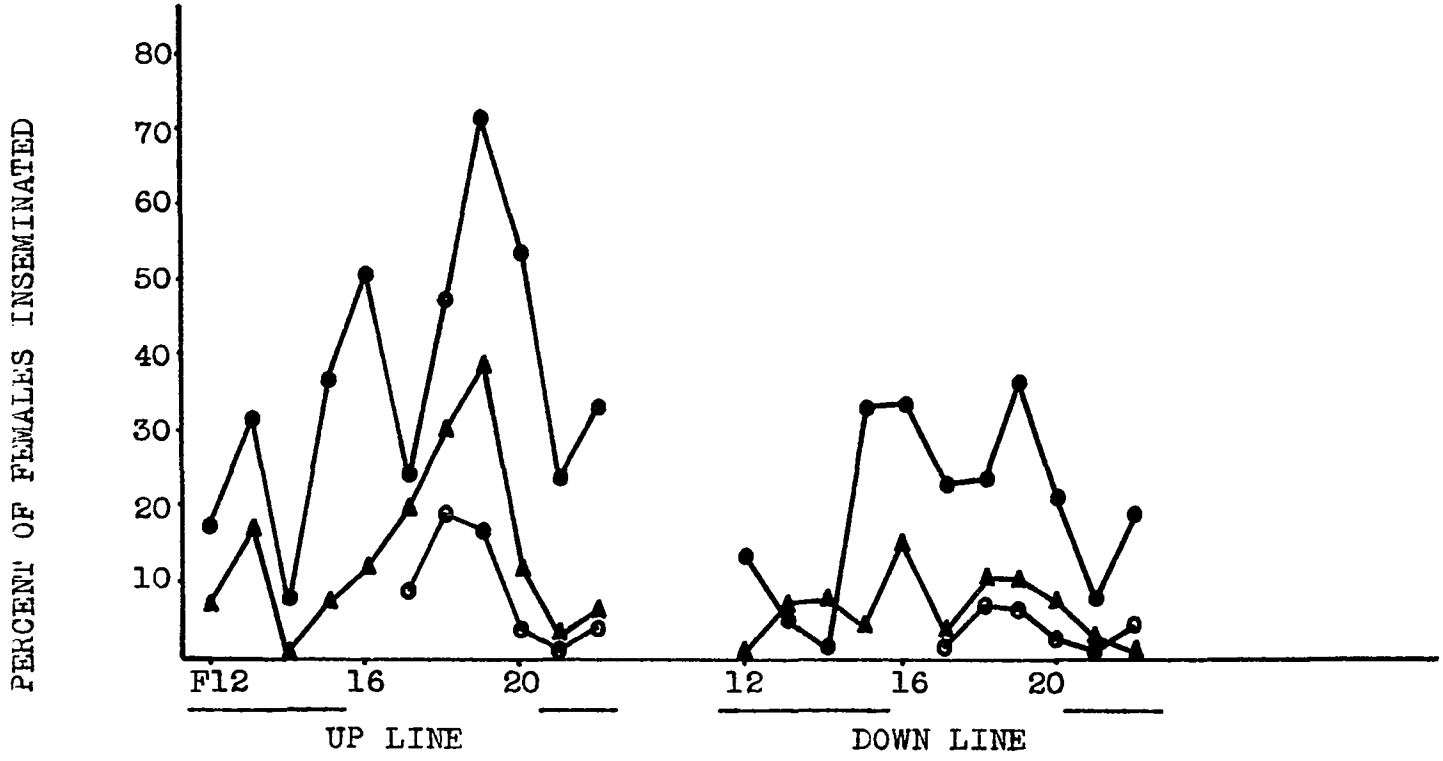


Figure 8. Female selection series 1. Percent of females inseminated for each generation in which two or one day matings were also set up. Underlined generations are in the April-Sept. interval.
● = 4 day matings, ▲ = 2 day matings, o = 1 day matings.

inseminated each generation) are placed into one of these two intervals and averaged, the results are as seen in Table VI.

Table VI

Average Percents of Females Yielding Hybrid Progeny

	UP LINE			DOWN LINE		
	one day matings	two day matings	four day matings	one day matings	two day matings	four day matings
April-Sept.	2.21	6.70	15.86	1.83	3.14	8.53
Oct.-March	11.11	22.26	29.87	3.56	9.74	17.35

Because of the marked seasonal effect on success of hybridization, I also regressed the transformed differences between the up and down lines on number of generations of selection within each of the two six month intervals (see Appendix, 3). The results for each six month interval indicated that a large and significant portion of the variance of the difference between the two lines was explained by regression on number of generations of selection. Although the slope was greater for the October-March interval ($b = 2.871$) than for the April-September interval ($b = 2.074$), the difference between the two slopes was not significant. Nonetheless, there is at least an indication that selection was more effective in the October-March interval. During this interval, of course, it was possible to select, as parents of the next generation in the up line, relatively more females which had mated with simulans males after just one or two days, thereby increasing the intensity of selection.

It should also be pointed out that even within a six month interval there are large fluctuations from one generation to the next. The causes of such fluctuations generally act on both lines in like manner, however, so that increases (or decreases) in mating in one line are accompanied by increases (or decreases) in the other. This is more apparent in the four day matings (Figure 6).

It is interesting to note that in the case of the one-day matings, in which inseminations are very few in number, all of the generations characterized by significant differences between the two lines fall into the October-March interval, during which matings more readily take place. With certain experimental designs, then, it is possible that genetic differences could be detected only at certain times of the year. Unfortunately, it is usually impossible to determine from the information given in the literature when certain experiments were done.

The results of female selection series two are seen in Figures 9 and 10 and Table VII. The response to twelve generations of selection is verified by significant differences (in the expected direction) between the two lines in the F-5, 7, and 11, and in all generations the percentage of inseminations was higher for the up line than for the down line. There were no significant differences between the two lines for any of the two day matings (Figure 9), although the differences were always in the expected direction. (The two day matings which showed

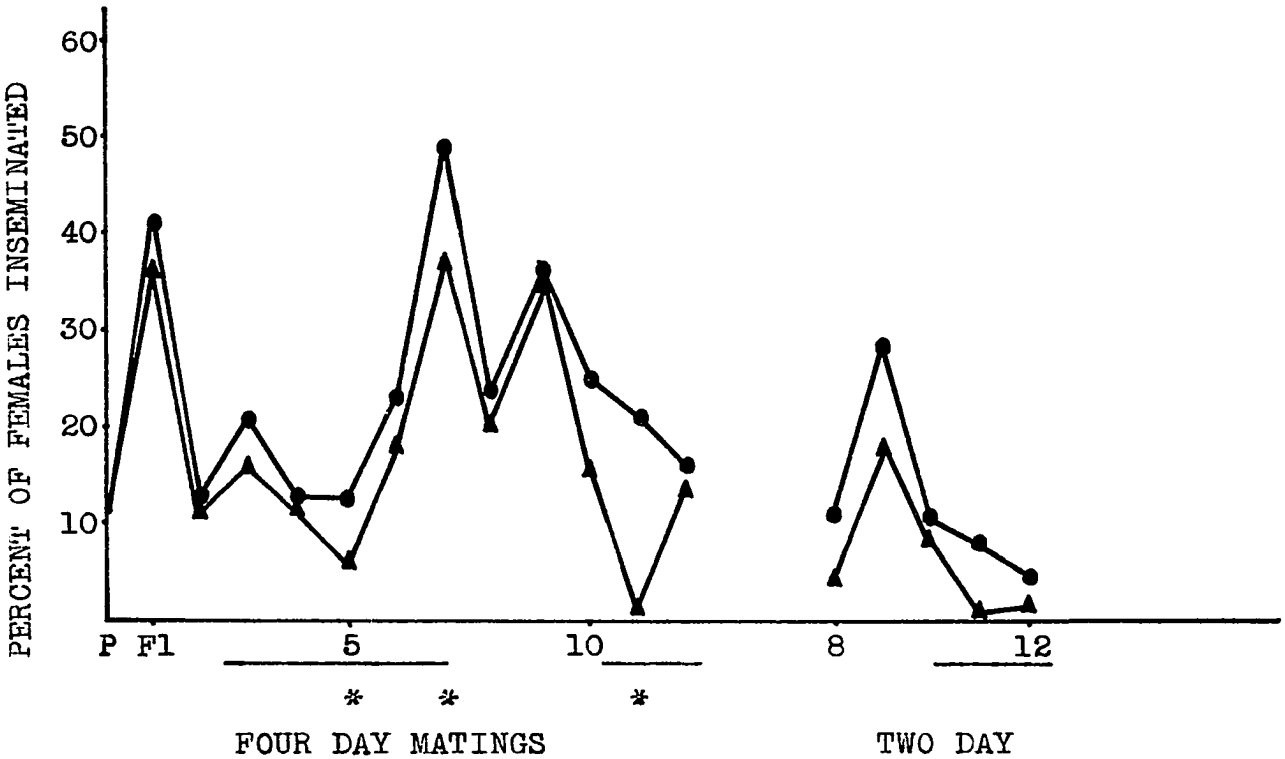


Figure 9. Female selection series 2. Percent of females inseminated for four and two day matings. Underlined generations are in the April-Sept. interval. Starred generations show significant differences in the expected direction. ● = up line, ▲ = down line.

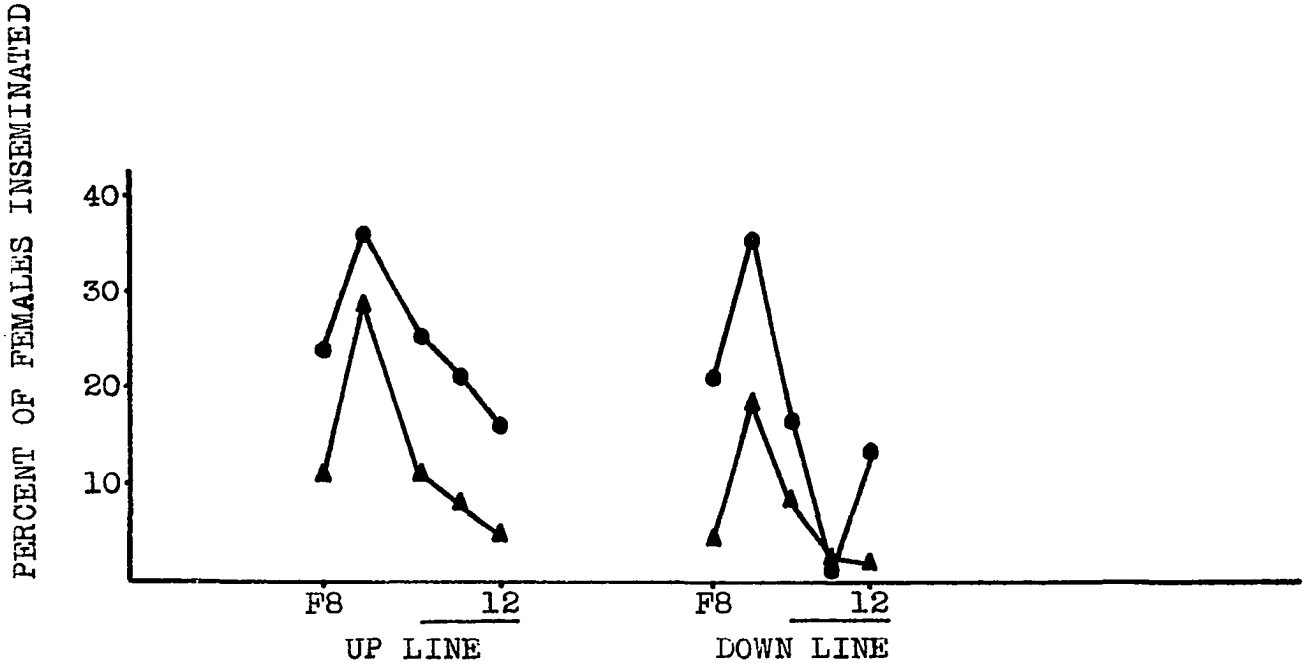


Figure 10. Female selection series two. Percent of females inseminated for each generation in which two day matings were also set up. ● = four day matings, ▲ = two day matings. Underlined generations are in the April-Sept. interval.

Table VII

Results of Female Selection Series 2

Generation		dates	UP no. of 5 pr. vials	UP no. of female tests	UP % in-semi-nated	DOWN no. of 5 pr. vials	DOWN no. of female tests	DOWN % in-semi-nated
P		1-1969	39	188	11.17	39	188	11.17
F-1		2-1969	36	174	40.80	36	177	36.72
F-2		3-1969	32	150	12.67	32	158	11.39
F-3		5-1969	28	139	20.86	28	131	16.03
F-4		7-1969	28	139	12.95	29	142	11.97
F-5		8-1969	34	166	12.65	34	167	5.99
F-6		9-1969	43	213	23.00	43	207	17.87
F-7		11-1969	39	193	49.22	39	189	37.04
F-8	2-day	12-1969	20	100	11.00	24	116	4.31
	4-day		20	96	23.96	24	120	20.83
F-9	2-day	1-1970	23	110	29.09	24	120	18.33
	4-day		24	117	35.90	24	119	35.29
F-10	2-day	3-1970	26	130	10.77	26	128	8.59
	4-day		25	123	25.20	25	124	16.13
F-11	2-day	4-1970	23	114	7.89	23	114	1.75
	4-day		25	123	21.14	25	124	1.61
F-12	2 day	5-1970	22	108	4.63	22	107	1.87
	4-day		22	107	15.89	22	110	13.64

significant differences in series one were not even begun until the F-12).

When I performed a regression analysis on the transformed differences between the two lines, I found that an insignificant portion of the variance of the difference between the two lines was explained by regression on number of generations of selection (see Appendix, 4). This is despite the fact that differences between the two lines are found two generations earlier than in series one (F-7). The results of regression analysis are particularly difficult to interpret, however, because of the large fluctuations in frequency of hybridization from one season to the next. More will be said in the Discussion about the various regression analyses.

Here again there is a reduction in hybridization in the summer months (indicated by the underlined generations on the horizontal axis of Figure 9). Averaging the results for the two lines for the two times of year for the two mating intervals gives the results seen in Table VIII.

Table VIII

Average Percents of Females Yielding Hybrid Progeny

	UP LINE		DOWN LINE	
	two day matings	four day matings	two day matings	four day matings
April - Sept.	6.26	17.75	1.81	11.19
Oct. - March	16.92	31.29	10.41	26.23

Again, as in female selection series one, fluctuations are generally parallel in the two lines. The influence of the

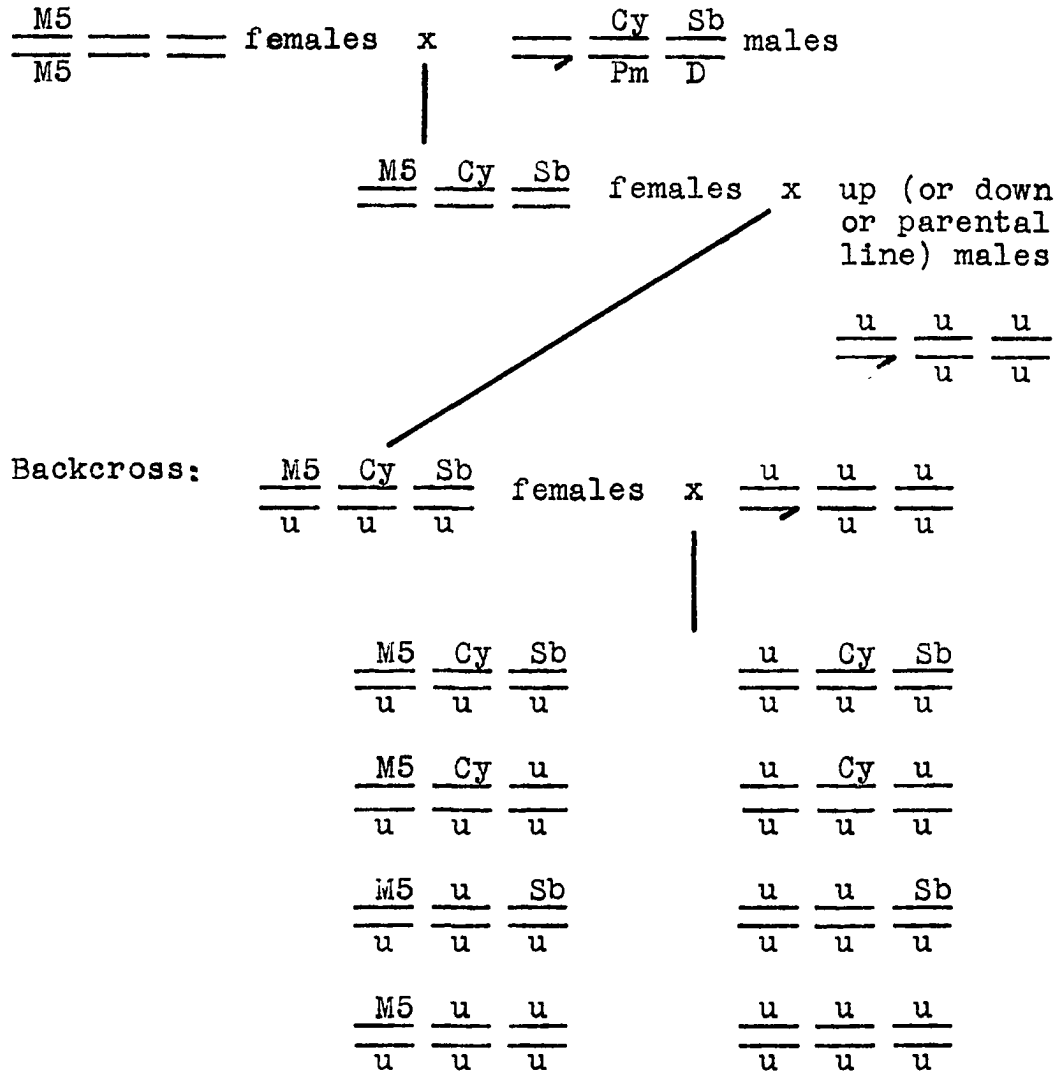
time available for mating is shown in Table VII and in Figure 10, where the two and four day matings are compared for both lines.

2. Genetic Analysis

a. Methods and Materials. To investigate the parallel effects of selection in the two female lines, as well as to possibly verify the polygenic nature of the traits being selected for, the following crosses were set up using female selection series one F-23 or 24 flies and series two F-13 or 14 flies: (1) up series one females x up series one males, (2) down series one females x down series one males, (3) up series one females x up series two males, (4) down series one females x down series two males, (5) up series one females x down series one males. In addition, a control culture (from the series one parental stock) was set up. Females from all six crosses were placed with stock simulans males for four days exactly as in the female selection experiments and then placed singly in vials in order to determine the percentage of females inseminated.

Using flies of the F-22 and F-23, the chromosomes of the series one female selection lines (up and down) were assayed for their respective contributions to the selected traits. This involves the use of dominant markers and inversions which act as crossover suppressors so that the selection line chromosomes will remain intact. The preliminary crosses are as shown, with only the three major pairs of chromosomes indicated;

contributions by the small fourth pair of chromosomes are ignored.



The yellow Muller-5 (M5) or Bascy chromosome (an X chromosome) is so called because it carries the incompletely dominant Bar-eye gene (B), the recessive white-apricot allele (w^a), the recessive scute-eight allele (sc^8), and the recessive yellow gene (y), as well as two inversions, $In(1)sc^{S1L}sc^{8R}$

and In(1)S, which act as crossover suppressors. The scute (sc) alleles cause a reduction in number of the scutellar and other bristles. With regard to chromosome II, the dominant Cy (Curly) gene causes curly wings and is associated with the inversion In(2L)Cy. The dominant Stubble gene (Sb) causes short, heavy bristles and is associated with a cross-over suppressor according to R. Runge (Lake Forest College) who sent me the stock. These dominant markers, associated with inversions, allow one to detect the presence of just one such chromosome since the phenotype of the marker appears when it is present in heterozygous condition, and all of the autosomal markers shown (Cy, Pm, D, Sb) are lethal in homozygous condition. The Bar eye gene (B) is not lethal in homozygous condition but can be recognized in heterozygous or homozygous condition by the shape and size of the eye.

The eight classes of females resulting from the last cross above are then placed with simulans males exactly as in the female selection experiments and later placed singly in vials in order to determine the percentage inseminated. By making the appropriate comparisons, one can determine the relative contributions of the three major chromosomes toward increased (or decreased) hybridization. The experiment was repeated using Dichaete (D) instead of Stubble (Sb). Dichaete causes the wings to be extended from the body axis and is associated with the inversion In(3LR)DcxF. At this time the chromosomes of the parental (unselected) stock were also assayed in the same fashion.

b. Results. The results of the various crosses between and within the female selection lines can be seen in Figure 11 and Table IX. The designations are as follows:

Table IX

Results of the Crosses Between and Within the Female Selection Lines (7-29-1970 to 8-9-1970)

cross	no. of females tested	% inseminated
U1 x U1	144	18.06
U1 x U2	156	12.82
U1 x D1	148	10.14
unselected	149	8.72
D1 x D1	145	21.38
D1 x D2	151	19.20

U1 x U1 = up series 1 females x up series 1 males, U1 x U2 = up series 1 females x up series 2 males, U1 x D1 = up series 1 females x down series 1 males, unselected = flies of the parental or base stock from which series one selection was begun, D1 x D1 = down series 1 females x down series 1 males D1 x D2 = down series 1 females x down series 2 males.

As seen in Figure 11, the parental line (unselected) and U1 x D1 crosses give almost identical results. This may be as expected if the selection lines carry "up" and "down" genes which "cancel out" in the hybrids.

The U1 x U1 results show significantly more frequent hybridization than the unselected cross, which is to be

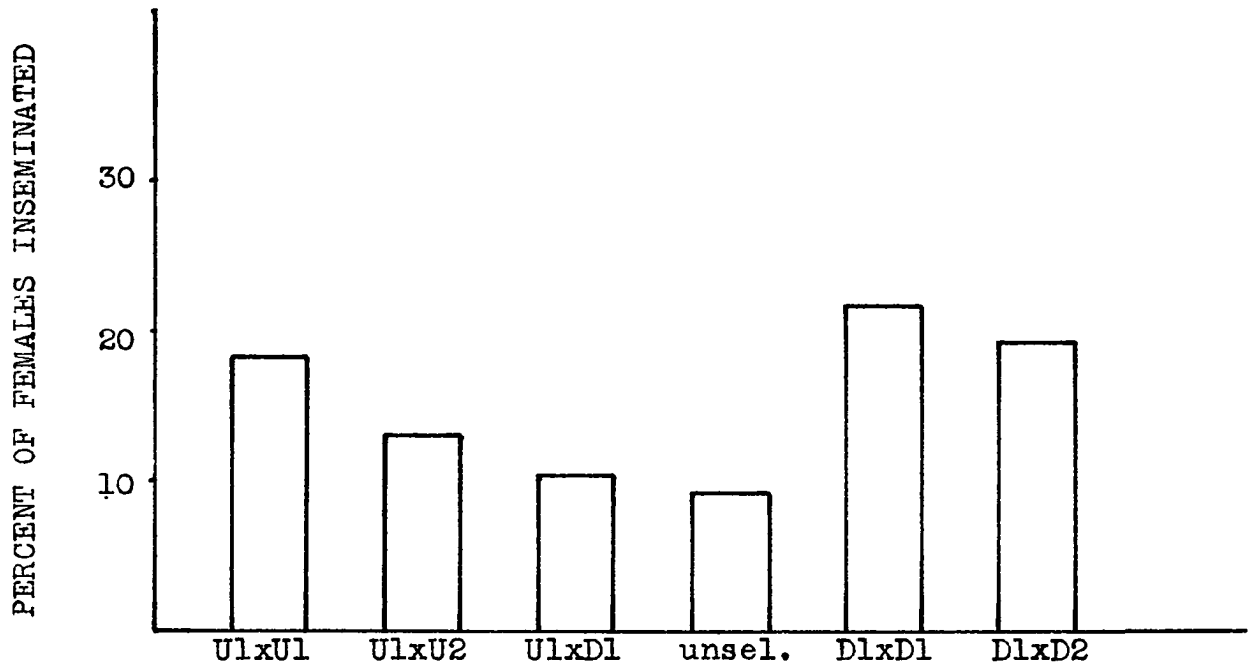


Figure 11. Percent of females inseminated among the offspring from crosses between and within the female selection lines.

expected if selection was effective in the "up" line. The U1 x U2 cross showed more hybridization than the unselected but not significantly more. A possible explanation is that the series 2 line was not selected for as long in the "up" direction as the series 1 line, and hybridizing the two lines may have diluted somewhat the "up" genes of the up series 1 line.

Both the D1 x D1 and D1 x D2 results appear anomalous in that they are like those of the U1 x U1 cross. A possible explanation of these results will be given below, after examining the results of the first chromosome assay.

The results of the first chromsome assay are shown in Table X. Approximately 100 of each of the eight classes of females in each of the two lines were tested with wild type simulans males to determine the relative frequencies of hybridization for all classes. To determine the contributions of the three major chromosomes to the selected traits, one must first determine the difference in frequency of hybridization between each pair of classes which differ in their chromosome makeup by just the one chromosome whose effect is being studied; the difference is presumably due to that chromosome. An average of such differences will then give an estimate of that chromosome's contribution to the trait in question. For example, to determine the contribution of chromosome III to increased hybridization in the up line, one finds that the following pairs of classes differ by just one up line chromosome III: 2 and 1, 4 and 3, 6 and 5, 8 and 7. An average of the

Table X

Results of the First Chromosome Assay
(7-10-70 to 7-23-70)

female class	no. of selected chromosomes	UP LINE no. inseminated / total	%	DOWN LINE no. inseminated / total	%
1. M5 Cy Sb	3	1/97	1.03	4/94	4.26
2. M5 Cy --	4	7/100	7.00	1/100	1.00
3. M5 -- Sb	4	5/101	4.95	2/84	2.38
4. M5 -- --	5	8/96	8.33	5/99	5.05
5. -- Cy Sb	4	3/100	3.00	0/90	0.00
6. -- Cy --	5	10/102	9.80	5/90	5.56
7. -- -- Sb	5	5/105	4.76	7/97	7.22
8. -- -- --	6	10/103	9.71	1/99	1.01
total		49/804	6.09	25/753	3.32

differences for these four pairs of classes would be found from the formula:

$$III = \frac{(2-1) + (4-3) + (6-5) + (8-7)}{4}$$

The above is a standard procedure for localizing the genes involved in some trait and is described by Hirsch (1967). When such values are calculated for each of the three chromosomes in both the up and down lines, the results are as seen in Table XI. Notice that the greatest divergence between the two lines is found for chromosome III and that chromosome II contributes least to the divergence.

Table XI

Contributions of the Three Major Chromosomes to Hybridization

chromosome	up line	down line
I	.0149	.0028
II	.0173	.0121
III	.0528	-.0031

Further verification of the differences between the two lines can be found. Taking the data as a whole, 804 up line females, approximately equally distributed among the eight classes, yielded 49 matings, whereas 753 down line females, also approximately equally distributed among the eight classes, yielded only 25 matings. This is a valid comparison, since for any particular class, the females of the two lines

carry exactly the same number of selected chromosomes. (See Table X). This 2:1 ratio of the numbers of matings in the two lines is in good agreement with the last five generations (four day matings) of the selection experiment, in which the up line showed approximately two to three times the percentage of females inseminated in the down line. Exact comparisons of percentages would be valid for only those generations sampled in middle to late July because of the seasonal effect. These comparisons and others are made in Table XII. Also shown for comparison is that class in the chromosome assay (8) which carries all six major chromosomes from the selected line and should therefore be identical to the selection line flies. In the last column, classes which carry either five or six of their six major chromosomes from the selected lines are added together to show the effect on hybridization of a preponderance of chromosomes from either selected line.

Table XII

Comparison of the percents of females inseminated in the up and down lines (of female selection series 1) after various generations of selection and in various parts of the assay

	SELECTION		CHROMOSOME ASSAY		
	F-4	F-14	sum	class 8	classes 4,6,7,8
up line	6.52	6.93	6.09	9.71	8.13
down line	3.21	1.98	3.32	1.01	4.68

In Figure 12 the results of averaging all assay classes having the same number of selected chromosomes are shown.

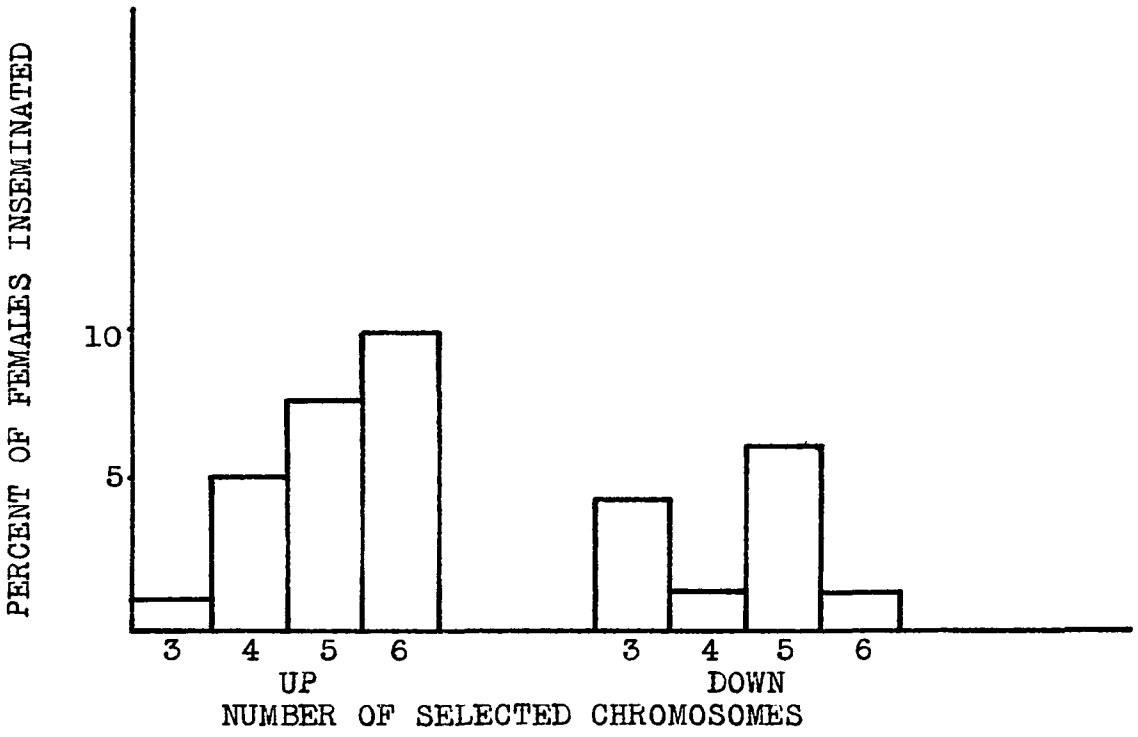


Figure 12. Percent of females inseminated in assay classes having the same number of selected chromosomes in the two lines (first assay).

Although an additive effect is apparent for the up line chromosomes, the effect of increasing the number of down chromosomes appears to be random.

To return to the apparently anomalous results of the inter- and intra-line crosses, the up and down line males which were used in the chromosome assay came from a generation of flies which had selected parents and would be expected to carry a sample of up or down line genes, respectively. However, the inter- and intra-line crosses, set up nearly a month later, involved flies at least a generation removed from selection. If the genes selected for increased hybridization in the up line were either additive and/or recessive in their effects on hybridization, one would expect, after 21 generations of selection, a degree of homozygosity (fixation) which would persist and continue to favor hybridization. This may have been the case as can be seen by comparing the U1 x U1 and unselected crosses of Figure 11. If, on the other hand, the genes which favored decreased hybridization in the down line were dominant in their effects, random mating would allow for the segregation of larger numbers of recessive homozygotes than in the last generations of the down line. This assumes that flies with the down phenotype are heterozygous for at least some of the dominant down genes (rather than homozygous for them all), and in this case random matings with recessive homozygotes (for a particular locus) would yield flies of which half are recessive homozy-

gotes, with no down phenotype, and half have a down genotype and phenotype. By contrast, when flies were selected for their down phenotypes, the matings presumably involved mostly heterozygotes or dominant homozygotes, and all or a majority of the offspring would have a down genotype for a particular locus. This may be shown as below:

Selected matings involve mostly the following:

(1) AA x AA (2) Aa x Aa (3) AA x Aa

Random matings could also include the following:

(4) AA x aa (5) Aa x aa (6) aa x aa

The frequency of occurrence of the various types of matings shown depends, of course, upon the gene frequencies and genotype frequencies, as well as the effect of the genotype upon intraspecific mating, at the time of last selection for the down phenotype. Judging from the rapidity of the rise in frequency of hybridization after selection was relaxed, it is likely that many flies at that time were heterozygotes for the down genes, so that matings of the type Aa x Aa and Aa x aa were common, or that in the population which resulted without selection of parents, flies with the down genotypes were selected against in "competition" for intraspecific matings.

A similar explanation may explain the high frequency of hybridization in the offspring from the D1 x D1 and D1 x D2 crosses.

This leaves us with the apparently anomalous results of the flies from the U1 x D1 cross. As mentioned earlier,

one might expect such flies to be identical to the unselected strain if the up and down genes "cancel out". However, it would be difficult to reconcile such results with the hypothesis proposed above regarding dominance of the down line genes and segregation of up phenotypes due to relaxation of selection. It should be remembered, however, that a phenotype is the result of the interaction of all of the genes in the genome or genetic background, and that the combination of up and down genes in the same individual may not give predictable results, perhaps due to gene interaction (which is unpredictable). The two lines of flies had become better adapted, after 21 generations of selection, to the artificial selection pressures involved, and this adaptation presumably involved not only an accumulation of genes directly involved in discrimination of foreign species but also selection for and against other genes indirectly involved in mating behavior (through sensory physiology, nerve action, etc.). The end result was two lines of flies with different, but harmonious, genetic makeups. The effects of hybridizing the two lines on the frequency of interspecific hybridization in the offspring are certainly not easy to predict for such a labile trait and one which involves an interaction between two individuals. It is also possible that artificial selection was for interacting genes and that a delicate gene balance governing species discrimination was destroyed in the U1 x D1 hybrids.

That the flies in the down line became genetically different (in the proposed direction) after artificial selection was relaxed is verified by the results of the second chromosome assay (Table XIII). The contribution of the three major chromosomes to hybridization are shown in Table XIV;

Table XIV

Contributions of the Three Major Chromosomes to Hybridization

chromosome	up line	down line	unselected
I	-.0179	.0017	.0559
II	.0690	.0268	-.0607
III	.0982	.1269	.1434

only classes with 50 or more females were used in the calculations. The up line chromosomes show the same relative order of importance in contributing to hybridization as in the first chromosome assay; that is, chromosome III is most important, followed by chromosome II, etc. This would tend to support the hypothesis that selection for recessive genes favoring hybridization had resulted in some genetic fixation. Although the up and down lines differed in the relative contribution of chromosome III in the first chromosome assay (in which the greatest difference between the lines was due to chromosome III), chromosome III now contributes most importantly to hybridization in both up and down lines. This would tend to support the hypothesis that selection in the down line was for dominant genes (in either heterozygous or homozygous

Table XIII

Results of the Second Chromosome Assay
(8-9-70 to 8-26-70)

female class	no. of selected chromosomes	UP LINE no. inseminated / total	%	DOWN LINE no. inseminated / total	%	UNSELECTED no. inseminated / total	%
1. M5 Cy D	3	0/21	0.00	0/53	0.00	0/26	0.00
2. M5 Cy -	4	2/53	3.78	10/82	12.20	5/50	10.00
3. M5 -- D	4	0/35	0.00	0/49	0.00	0/23	0.00
4. M5 -- -	5	12/68	18.75	16/77	20.78	8/61	13.11
5. -- Cy D	4	0/34	0.00	0/59	0.00	0/10	0.00
6. -- Cy -	5	6/67	8.95	16/94	17.03	15/54	27.78
7. -- -- D	5	0/48	0.00	4/51	7.85	0/33	0.00
8. -- -- -	6	8/90	8.89	10/116	8.63	4/62	6.45

genotypes), and that the relaxation of artificial selection was followed by natural selection for their recessive alleles (selected for artificially in the up line). The majority of the loci involved appear to be on chromosome III. That natural selection (in the down line) for intraspecific mating activity might cause a convergence in genetic makeup of chromosome III in the two lines is further supported by the important contribution to hybridization of chromosome III in the unselected line. In this unselected stock, natural selection for intraspecific mating activity has presumably been even stronger than in the up and down lines for the following reason: because relatively large numbers of flies were together each generation in the unselected stock, courtship may often have been interrupted by the activity of other flies for all but the most sexually receptive females; by comparison, relatively small numbers of females of the up and down lines were always placed with approximately equal numbers of male sibs into vials in which mating could more easily occur without interruption. It seems likely that the same recessive genes favor intra- and interspecific mating activity, and that the dominant alleles tend to inhibit both kind of mating activity.

With regard to the other major chromosomes, the down line chromosome I, which contributed less to hybridization than the up line chromosome I in the first chromosome assay, now contributes slightly more to hybridization than its up line

homolog. Possibly the same type of selection has operated in the case of chromosome I (as for chromosome III) but on fewer loci. It should be noted that chromosome I is also much shorter than chromosome III. However, the down line chromosome II, which is similar in length to chromosome III, contributed even less to hybridization in the down line in the second chromosome assay (when compared to its up line homolog) than it did in the first assay (when compared to its up line homolog). It is possible that few or no loci which significantly influence the traits selected for are on chromosome II.

That the up and down lines have converged since the relaxation of artificial selection is further supported by a comparison of the results of averaging all assay classes having the same number of selected (or unselected) chromosomes (Figure 13). Although all three lines show a similar additive effect, only the up line chromosomes showed such an effect in the first chromosome assay (in which the unselected stock was not studied). It should be noted that the sample sizes in the second chromosomal assay were smaller than in the first assay (because fewer flies eclosed in the preliminary crosses).

In light of the changes which may have occurred in the unselected and down line stocks due to natural selection since the beginning of the selection experiment, the results of the second chromosome assay cannot be used to determine whether selection was more effective in the up or down direction. However, the evidence for the recessive nature of the

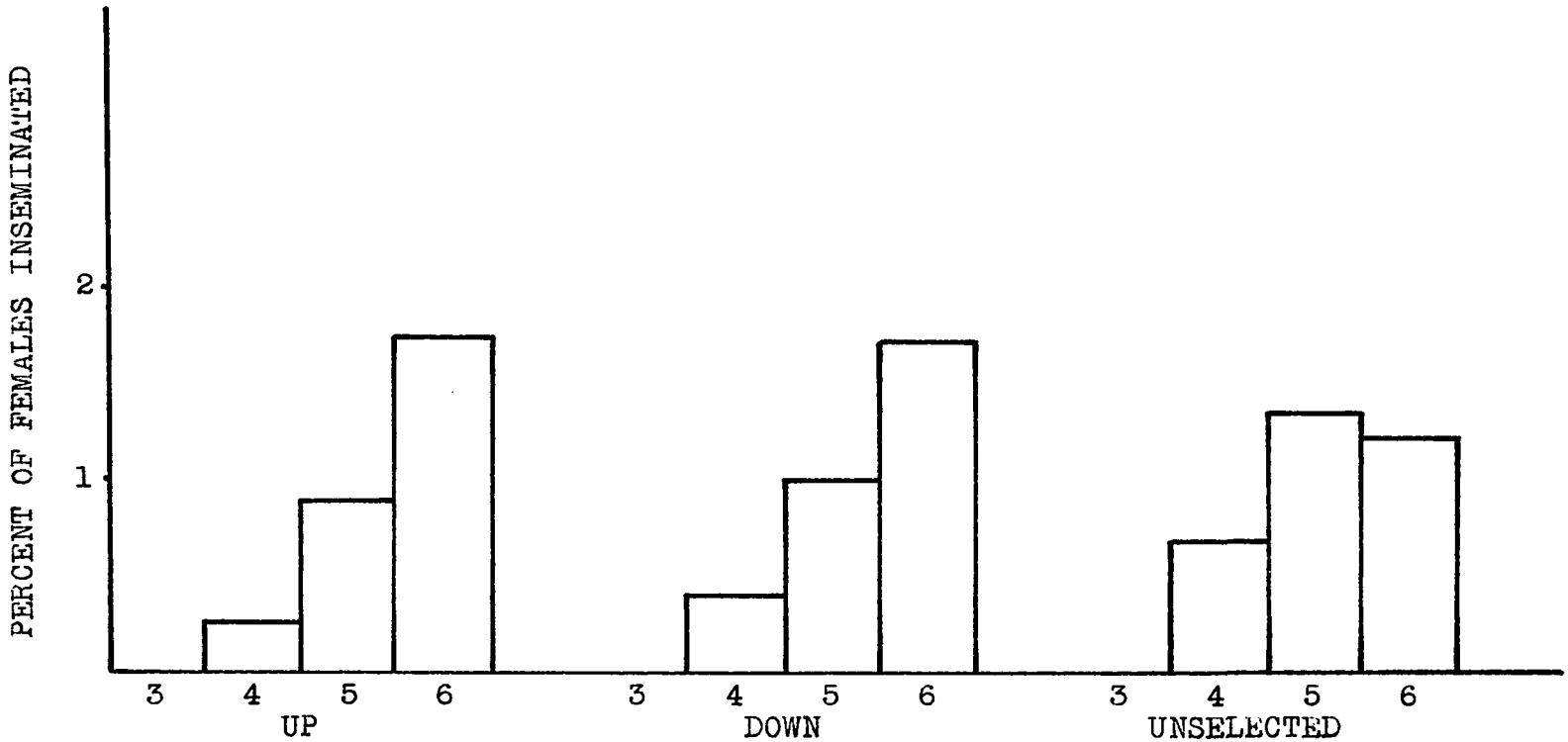


Figure 13. Percent of females inseminated in assay classes having the same number of selected chromosomes (second assay).

genes favoring hybridization would support the hypothesis that selection was more effective in the up direction. Evidence for the disadvantage of down line females in intra-specific matings will be presented in the next section. That up line females of assay class number 8 (with all up line chromosomes) do not show much more frequent hybridization with simulans males than unselected line females of assay class number 8 (with all unselected line chromosomes) may not be too surprising since the selected genes are probably becoming increasingly diluted with unselected genes.

3. Evidence for the Greater Attractiveness of Up Line Females and Observation of Courtship in Flies from Selection Lines

In the F-18 I placed up and down line females in the same vial with simulans males (for two or four days) in order to determine whether one type of female would be preferred in a male "choice" situation. Equal numbers of females of the two lines were marked with India ink on the dorsal part of the thorax so that they could be told apart at the end of two or four days, and the marking did not influence insemination since equal numbers of marked and unmarked females were inseminated. The results are seen in Table XV. Although the difference is not significant, it is in the direction to be expected if up line females are more attractive to simulans males.

Table XV

Up and Down Line Females Inseminated by *simulans* Males in a Choice Situation

line	inseminated	total	% inseminated
up	9	149	6.04
down	5	149	3.36

I also made observations on the behavior of up and down line females which were confined with stock *simulans* or *melanogaster* males. Mating in the genus *Drosophila* usually comes at the termination of a period of courtship during which the flies are very close together. The usual movements involved are as follows (Manning, 1959a): (1) tapping, in which the male taps the female's body with his foretarsi, after which courtship may begin; (2) orientation, in which the male faces the female and follows her; (3) male wing display, either scissoring (predominant in *simulans*) or vibration (predominant in *melanogaster*), during which the species-specific "love-song" is generated (Bennet-Clark and Ewing, 1970); (4) licking by the male of the female genitalia: (5) attempted copulation. The female may respond by standing still, by making movements which are inhibiting to courtship, such as extrusion of the ovipositor, or by producing a "song" of her own, the repulsion signal (Bennet-Clark and Ewing, 1970). I considered it possible that selection had acted in such a way as to visibly alter the courtship pattern, and the purpose of

the observational work was to discover which part of the courtship, if any, had been modified.

For the observational work, I used pairs of flies which were shaken (two or more hours after etherization) into the space beneath a 40 mm watch glass and observed through a dissecting microscope (low power). I reported all observations into a tape recorder and made all observations during the hour or two following midnight. I used females which were the offspring of selected parents and should therefore have retained the behavioral differences selected for. I made observations on nine different females of each line, and the order in which the flies of the two lines were observed alternated from day to day.

Since I thought it possible that females of the two lines differed in general activity levels, perhaps in such a way as to better enable the down line females to escape from the males, I decided to try to determine general activity levels in the two lines. In order to determine the general activity level of the flies being observed, I placed the watch glass over a note card marked off into one-centimeter squares and counted the number of squares a female entered during various one-minute intervals. In addition, I recorded the interval during which a female was being courted by the male; the time I spent observing a female was approximately the same for each line on any one day. The observation intervals ranged from three minutes thirty seconds to ten minutes thirty seconds on different days.

The experimental design for the observational work was as follows: I shook a female, of the up line for example, from a vial into the space under the watch glass, after which I shook a stock *simulans* male from a vial into the same space. After the observations were over, I retrieved the *simulans* male and replaced it with a *melanogaster* male. After the next set of observations, I discarded the up line female, retrieved the *melanogaster* male, and placed the same *simulans* and *melanogaster* males successively with a down line female. In this way, a *melanogaster* female was always exposed first to a *simulans* male and then to a conspecific male, and the two types of female were always exposed to the same males in order to avoid difficulties due to differences in male mating behavior. The females observed on any one day were always of the same age in order to avoid difficulties due to age differences in female sexual receptivity.

In order to determine whether observations of the same female would be at all similar from one day to the next, I observed the first two females of each line on each of two succeeding days. The data collected from these four flies (two of each line) are seen in Table XVI, in which the numbers in parentheses indicate the number of one-minute intervals which were averaged. It should be noted that *simulans* males never succeeded in courting either type of female beyond tapping or the initiation of courtship.

Table XVI

Behavior of Four Series 1 Female Selection Females

	average no. of 1 cm blocks entered during 1 min. intervals when confined with: simulans male on		melano. male on		percent of time courted when confined with mel. male	
female	day 1	day 2	day 1	day 2	day 1	day 2
up						
no.1	12.5(4)	13.2(4)	9.3(3)	10.7(3)	70.00	68.25
no.2	20.0(4)	16.0(3)	26.3(3)	16.0(3)	80.00	64.17
down						
no.1	15.2(4)	13.5(4)	24.3(3)	32.3(3)	77.50	75.00
no.2	40.5(2)	3.8(4)	18.0(2)	2.7(3)	9.17	0.00

It appears that although the activity level of the females may fluctuate considerably from one day to the next (see down female no.2), the percentage of the time that a particular female is being courted by a conspecific male is much the same from one day to the next.

For the nine females of each line the data collected may be summarized as seen in Table XVII (the numbers in parentheses indicate the number of one minute intervals averaged).

Table XVII

Behavior of Series 1 Female Selection Females

	average no. of 1 cm blocks entered during 1 min. intervals when confined with: simulans male	melanogaster male	average percent of time being courted when confined with melanogaster male (out of 64 minutes)
females			
up	26.8(34)	25.7(30)	57.84
down	26.3(34)	27.8(30)	37.47

Although, during the observations made, the simulans males never succeeded in courting either type of melanogaster female beyond tapping (the initiation of courtship), it does indeed appear that down line females are not so "attractive" to even melanogaster males as the up line females. Because only intraspecific courtships could be observed during a reasonable length of time, and because the results of genetic analysis indicate that the same genes which favor hybridization may also favor intraspecific mating, differences in relative "attractiveness" in intraspecific courtship between the females of the two lines may be taken cautiously as an indication of differences which may exist in interspecific courtships.

The differences in general activity levels (as measured by counting the number of one-centimeter blocks entered during one-minute intervals) appear too small to account for differences in frequency of hybridization, and, as seen in Table XVI, activity levels fluctuate considerably from one day to the next. Females are capable of giving repelling movements, such as extrusion of the ovipositor, to ward off courting males, but I observed such extrusions in both kinds of females. Because I was observing in such a way as to record when the male involved began or ceased to court and because I could observe extrusions only when the female was in a particular position, I did not make a count of repelling movements. It is therefore possible that females of the two

lines differ in the degree to which they repel courting males, either through overt behavior or through production of the repulsion signal. Chemical differences (as in species specific scents) between females of the two lines are also not ruled out.

B. Male Selection

1. Selection

a. Methods and Materials. In the male selection experiments pair matings were used since small mass matings would not allow determination of exactly which males had mated. For a few generations, males and females of the same age as in the above experiments were used, but difficulty in obtaining crosses led to the later use of one to two day old females (instead of three to four day old females), after which matings were regularly obtained and selection for decreased isolation made possible. Apparently the older females mate only in small mass mating vials where one courtship may stimulate others. For a short period of time, wild type simulans males were placed singly with two females; the same problem (nearly complete absence of matings) arose and was solved by using pair matings. The male is possibly more persistent in courting one female when no others are present than when another female is available. Flies were always left together for four days, and in the later generations one and two

day matings were also set up in an attempt to intensify selection in the "up" line; the experimental design for these additional matings was exactly the same as for the female selection experiment. After one generation of selection, selected males were mated with virgin female sibs in order to parent the next generation. Although the number of males and females used to parent the next generation varied from generation to generation, the number of female sibs was always about the same for up and down lines (and seldom fewer than ten) for any particular generation, in an attempt to maintain similar population densities in cultures of the two lines. Again, there was a degree of selection for intraspecific mating in both lines.

The male selection experiment was carried out using two stocks. One, for male selection series one, was a wild type lab stock which had been maintained by mass culturing for years (the same stock used in all of the environmental studies and the female selection experiments). The other, for male selection series two, was synthesized from the following eight wild type stocks: the lab stock and stocks received from Pasadena, Oak Ridge, University of Umea, Sweden (two), and Amherst College (three). The heterogeneous stocks were made in the same way as for female selection series two.

b. Results. The results of series one are seen in Figure 14 and Table XVIII. There are no significant differences between up and down lines after nine generations of

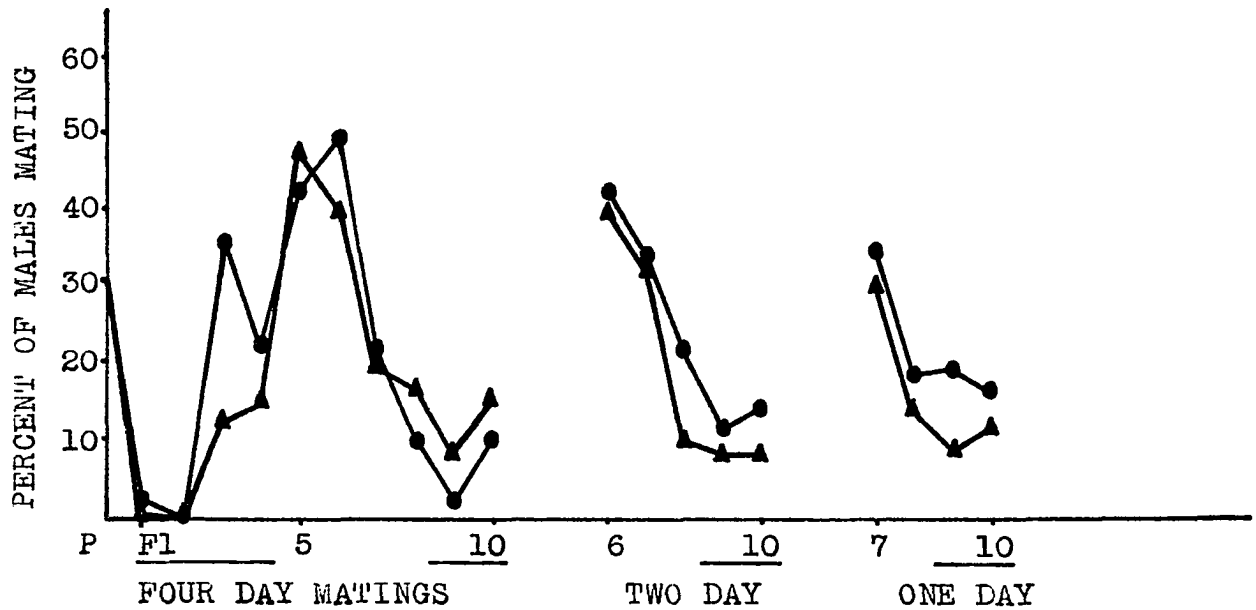


Figure 14. Male selection series 1. Percent of males which mate for one, two, and four day matings. Underlined generations are in the April-Sept. interval. ● = up line, ▲ = down line.

Table XVIII

Results of Male Selection Series 1

Generation		dates	UP no. of crosses	UP % with offspring	DOWN no. of crosses	DOWN % with offspring
P	(using 3-4 day females in pr.matings)	3-1969	171	29.82	171	29.82
F-1	(using two 3-4 day females with 1 male)	5-1969	140	0.00	141	0.00
	(using 3-4 day females in pr.matings)		82	4.88	87	2.30
F-2	(using 3-4 day females in pr.matings)	7-1969	122	0.00	125	0.00
F-3	(using 1-2 day females in pr.matings in F-3 through F-10)	8-1969	14	35.71	8	12.50
F-4	4-day	9-1969	89	22.47	93	15.05
F-5	4-day	11-1969	87	42.53	91	48.35
F-6	2-day	12-1969	59	42.37	59	38.98
	4-day		112	49.11	115	40.00
F-7	1-day	1-1970	93	34.41	93	30.11
	2-day		90	34.44	93	33.33
	4-day		36	22.22	36	19.44
F-8	1-day	3-1970	44	18.18	43	13.95
	2-day		50	22.00	49	10.20
	4-day		51	9.80	49	16.33
F-9	1-day	4-1970	80	18.75	80	8.75
	2-day		86	11.63	89	7.86
	4-day		59	1.69	57	8.77
F-10	1-day	6-1970	49	16.33	50	12.00
	2-day		57	14.04	58	6.20
	4-day		75	10.67	71	15.49

selection, and, for the four day matings, the lines cross frequently. No selection in the up line was possible in the F-2 since no matings were obtained, and various alterations in experimental design were made in the early part of the experiment. (See a. Methods and Materials). However, beginning with the F-3, the design was the same for the remainder of the experiment.

The seasonal effect is seen here also. The generations underlined on the horizontal axis fall into the April through September interval, and the peaks occur during the rest of the year. Only the F-4 and later generations should be examined critically since the experiment was altered from time to time in the earlier generations, and the F-3 sample was extremely small. When the percentages of males which inseminate females are averaged for all generations (after the F-3) within the same six month interval, the results are as seen in Table XIX. There were only three generations with one day matings, so that these are not well sampled in the two intervals. The two day matings are averages of only two or three samples, but the figures for four day matings are averages of four or six generations and are more reliable for comparison.

Again, as in female selection fluctuations are generally parallel in the two lines. As can be seen from Figure 14, there is no increase in matings with longer time intervals in these pair matings. The factors determining the success of a courtship apparently operate within 24 hours.

Table XIX

Average Percents of Males Which Mate (Series 1)

	UP LINE			DOWN LINE		
	one day matings	two day matings	four day matings	one day matings	two day matings	four day matings
April-Sept.	17.54	12.83	11.61	10.38	8.24	13.10
Oct.-March	18.18	32.94	30.92	13.95	27.51	31.03

The results of male selection series two are seen in Figure 15 and Table XX. The early divergence between up and down lines is striking, and there are significant differences between lines in the F-3,4, and 6 for the four day matings, in the F-3, 5, and 6 for the two day matings, and in the F-4, 5, and 6 for the one day matings. A regression analysis indicated that an insignificant portion of the variance of the difference between the two lines was explained by regression on number of generations of selection (see Appendix, 5). More will be said in the discussion about the various regression analyses.

Again, the fluctuations are usually parallel in the two lines, and the peaks are during the October through March interval. Table XXI shows the averaged results for the various matings for the two six-month intervals. It should be kept in mind that the various types of matings were sampled only once during the April-September interval.

As in the other male selection experiment, there is no increased frequency of mating in the longer two and four day intervals when compared to the one day matings.

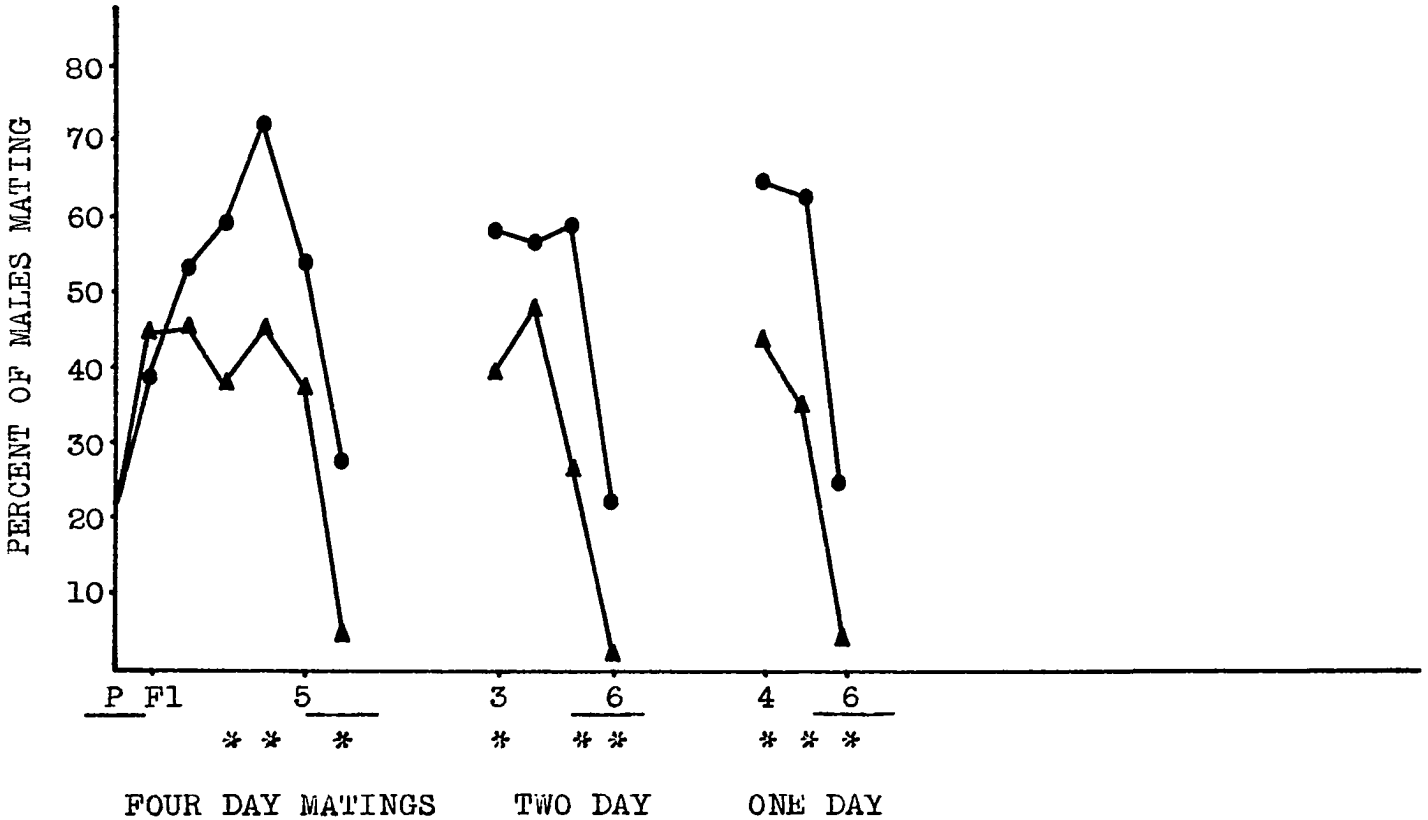


Figure 15. Male selection series 2. Percent of males which mate for one, two, and four day matings. Underlined generations are in the April-Sept. interval. Starred generations show significant differences in the expected direction. ● = up line, ▲ = down line.

Table XX

Results of Male Selection Series 2

Generation	dates	UP no. of crosses	UP % with offspring	DOWN no. of crosses	DOWN % with offspring
P 4-day	9-1969	185	22.16	185	22.16
F-1 4 day	10-1969	83	38.55	91	45.05
F-2 4-day	11-1969	79	53.16	79	45.57
F-3 2-day	1-1970	60	58.33	63	39.68
4-day		61	59.02	63	38.10
F-4 1-day	2-1970	48	64.58	48	43.75
2-day		46	56.52	48	47.92
4-day		46	71.74	46	45.65
F-5 1-day	3-1970	65	63.08	73	35.62
2-day		73	58.90	74	27.03
4-day		65	53.85	69	37.68
F-6 1-day	5-1970	80	25.00	81	3.71
2-day		82	21.95	12	2.44
4-day		66	27.27	64	4.69

Table XXI

Average Percents of Males Which Mate (Series 2)

	UP LINE one day matings	UP LINE two day matings	UP LINE four day matings	DOWN LINE one day matings	DOWN LINE two day matings	DOWN LINE four day matings
April-Sept.	25.00	21.95	27.27	3.70	2.44	4.69
Oct.-March	63.83	57.92	55.26	39.68	38.21	42.41

2. Observation of Courtship in Flies from the Selection Line

Using *simulans* males of the series two selection experiment, I made observations similar to those made for the female selection females (p. 89). I used males of the F-8 (or later) generation after selection had been relaxed for a generation or more. The results of observations on eight males of each of the two lines are seen in Table XXII (numbers in parentheses indicate the number of one-minute intervals which were averaged).

Table XXII

Behavior of Series 2 Male Selection Males

males	Average no. of 1 cm blocks entered during 1 min. intervals when confined with: *melanogaster* female	*simulans* female	Average percent of time courting when confined with a *simulans* female (out of 62 minutes)
up	19.5(28)	29.6(15)	18.95
down	20.4(28)	31.7(14)	1.74

Although the *simulans* males (of either line) are much more active when confined with a conspecific female, the difference in activity between the two types of male when confined with a particular type of female appears to be too small to account for the difference in frequency of hybridization between the two lines. Since, during these observations, up and down line males showed approximately the same extremely

small amount of courtship to melanogaster females (50 and 40 seconds, respectively, during 62 minutes of observation), the difference between the two lines in the percentage of time spent courting a conspecific female may possibly be taken as an indication of behavioral differences which might result when these males are confined for long periods (one, two, or four days) with foreign (melanogaster) females. The up line males spent more than ten times as much time courting a simulans female as did the down line males, and I observed no other difference in male courtship between the two lines. It is possible , of course, that selection has also altered species-specific odors or the nature of the simulans male's "love-song".

It would seem that male selection, like female selection, has altered both intraspecific and interspecific mating behavior in the same direction (toward greater mating activity, whether intraspecific or interspecific). Although this result may have been achieved through passive changes in flies in the female selection experiment, possibly through greater female receptivity or through the development of odors stimulating males to court, it has possibly been achieved in the male selection experiment through a difference in active courtship behavior. Whether the up or down line has been most affected by selection cannot be determined since the parent (unselected) stock was lost and unavailable for comparison.

C. Discussion

With regard to selection in melanogaster females for altered isolation between the two species, it is clear that female discrimination has a genetic basis, because of the response to selection, and is a polygenic trait, with most of the loci involved (in the differences between the up and down lines of series 1) located on chromosomes I and III. According to Broadhurst (1960), the mere fact that selection for a trait is possible is strong presumptive evidence that the trait has a hereditary determination. That differences between individual chromosomes (of the up and down lines) were in the expected direction (in the first chromosome assay, before the relaxation of selection) is further confirmation of the genetic basis of female discrimination as is also the indication of successful selection (with just twelve generations) in a replicate line (female selection series 2). That differences between up and down lines in female selection series 2 were found earlier than in series 1 may be expected from the greater genetic variability of the stock synthesized for series 2 selection. Perhaps a newly synthesized stock (series 2) contains some genetic variability which can be more rapidly exploited through the selection of genomes resulting from simply the independent assortment of the chromosomes from the various parental lines. The two lines do differ, though not significantly, in the rate of increase of difference

between the up and down lines (see Appendix, 5) with series 2 showing less increase with time.

According to Mather (1941), selection from inbred lines is very much less effective than from crosses between parental lines, and Carson states (1958) that if genetic variability is introduced by outcrossing before selection, dramatic responses may be observed. It should be remembered, however, that the outcrossing involved in deriving the series 1 female selection stock from the multiple sex-linked marker stock (y ct^6 ras f) undoubtedly introduced some genetic variability into this stock, thereby explaining some of the success of selection in female selection series one.

It may be considered that selection was more effective in the up line (of female selection series one) than in the down line. A response to selection is said to be asymmetrical (Robertson, 1955) if it is more marked in one direction than another. The Ul x Ul intraline cross yielded about twice the percentage of inseminations found in the unselected stock, and this was approximately the difference in magnitude between the up and down lines during the last five generations. One might expect less successful selection for decreased hybridization, since natural selection has undoubtedly acted in this direction and caused the accumulation of many genes favoring discrimination. This is supported by the fact that hybrids are extremely rare in nature (Mourad and Mallah, 1959) even though the two species are sympatric in at

least part of their distributions (Erk and Sang, 1966; Manning, 1959b; Moore, 1952). Hybrids are also very rare in population cages with large numbers of the two species (Barker, 1962b).

The first chromosome assay indicated that the genes for increased hybridization are additive in their effects, and a combination of results from the chromosome assay and from the intraline crosses (D1 x D1) made it seem likely that the genes for decreased hybridization are dominant (non-additive). More effective selection in the up direction is not surprising in light of the additive (possibly recessive) nature of the up alleles. Fulker (1966) discovered some dominance for genes for high mating speed and stated that most of the advance in selection for high and low mating speed should be for slow mating, just as Manning had found (1961).

The second chromosome assay helped to confirm the hypothesis that the relaxation of artificial selection had allowed natural selection, in the down line, for the recessive alleles at the "mating behavior loci". That the females of the down line might indeed be at a disadvantage in "competition" for intraspecific matings was verified by the results of observations of individual females.

Selection for altered isolation due to changes in male behavior was also effective. The effect of initial genetic variability is particularly clear in this case, since the series 1 stock, which showed no response to selection, had

been mass cultured for generations in the laboratory, and selection pressures may well have caused considerable homozygosity (genetic fixation) for loci influencing adaptation to this particular environment. In addition, such a shelf stock may often have undergone genetic drift due to the small numbers of flies involved in some transfers. By contrast, the synthesized stock showed the most rapid response to selection of any employed in selection. Observations of individual males from the series 2 lines indicated that down line males may have a disadvantage in "competition" for intraspecific matings and, by implication, in interspecific matings as well.

Possible reasons for differences in degree of response to selection are likely to be further elucidated by some discussion of the causes of phenotypic variability. The traits selected for ("reluctance" and "willingness" to hybridize) are very labile, as emphasized in the Introduction, but only those genetic differences whose effects are completely expressed in the phenotype are subject to the full rigor of selection and expected to give immediate response (Mather and Harrison, 1949). For traits in which selection favors homozygosity, further phenotypic variability may result from the generally lower degree of stabilization of homozygous flies against environmentally caused changes (Maynard Smith, 1958). In addition differences in phenotype produced by a change at one locus may be greater against one genetic background than

another, and genes which are selected due to heterotic (rather than additive) effects will always result in segregation of non-selected genotypes (and phenotypes) (Maynard Smith, 1958). Considering the different genomes which must have existed in the different stocks prior to selection, the differences in response are not surprising.

The results of the selection experiments are in general agreement with other attempts to alter sexual isolation or mating behavior by selection. Kessler (1966) selected for increased and decreased isolation between D. pseudoobscura and persimilis in both males and females of each species. In the pseudoobscura selection experiments, he found that separation of decreased and increased isolation lines in the female selection line was apparent by the F-5 and that female selection responded faster than male selection (F-9). In the persimilis selection lines, selection for decreased isolation was equally rapid in either sex, but selection for increased isolation was effective in only the female line. Since, in all of his selection lines, there was also selection for rapid mating (by the males or females) with the opposite sex of their own species (in order to parent the next generation), he concluded that heterospecific mating activity and conspecific mating activity are genetically different traits, because of the success in selecting either for increases in both, or for simultaneous increase in one (conspecific mating activity) and decrease in the other (heterospecific mating activity).

The only other example of selection for or against heterospecific mating is the work of Koopman (1950), who started population cages with both pseudoobscura and persimilis and obtained a big reduction in hybridization by removing the hybrids each generation. Other examples of alteration of mating behavior by selection were discussed in the Introduction.

Although selection in only one sex is expected to be more difficult than selection in both sexes, because the selected genes are diluted somewhat each generation, examples of successful selection for mating behavior traits using one sex only have been found and were discussed in the Introduction. One-sexed selection for morphological traits can also be successful; Frankham (1968) selected for abdominal bristle number in males or females only and found selection to be equally effective either way. Of course, the failure to obtain a desired response to selection in one experiment would not prove the lack of a genetic basis (Hirsch, 1962), and Hirsch further reports that replicate experiments don't always give comparable results (in Dobzhansky's lab).

There is a number of ways in which genetic changes may have altered mating behavior. It is possible that selection has caused an increase or decrease in reactiveness to disturbing stimuli (other flies), resulting possibly in decreased and increased frequencies of mating, respectively (Manning, 1961). Since simulans males have a slower rise of

excitation (readiness to mate) in courtship than melanogaster males (Manning, 1959a), it may be that thresholds of response were altered in the male selection experiments, perhaps through changes in membrane permeability and enzyme secretion in neurons (Manning, 1967b). If courtship functions as an isolating mechanism, it is possible that quantitative variations in courtship are responsible for the changes observed. It is possible that chemoreceptors have been altered or that the intensity of species-specific scents has been changed. Scents may even have been qualitatively changed; Manning (1967b) states that chemical differences are likely to arise early in divergent populations and that no other group shows such sensory divergence between such close relatives as does the genus Drosophila.

The success in selection for altered isolation in both sexes verifies that each sex plays a role in hybridization. The greater role of one sex or the other in mate choice in the genus Drosophila has its proponents, however, depending often upon which species or aspects of mating are emphasized. Carmody et al. (1962) state that the choice of mates in the genus Drosophila is determined mainly by the female. Bateman (1949) considers the determining factor in mate choice to be the degree to which females repel the males, based upon Streisinger's findings (1948) that certain interspecific matings (see Introduction) are at random when the females are etherized. Merrell (1954) considers that, within species or among

closely related species (of Drosophila) the female determines whether copulation occurs. Other evidence for the female role is available (Bateman, 1948; Merrell, 1949 a,b).

The male role in mate choice is emphasized by King (1947), who found that for the guarani species group sexual isolation is largely a function of male behavior. Spieth (1951) found that in the virilis species group, both sexes are responsible for sexual discrimination, but that it is primarily a function of the male. Parsons (1965) found that for five pure (inbred) stocks of melanogaster, the stock of origin of the male influences the duration of mating between the stocks. Kaul and Parsons (1965) found that mating speed (in pseudoobscura) is determined mainly by the male karyotype (for various combinations of standard and Chiricahua gene sequences).

Evidence for the role of both sexes in mating comes from Merrell (1949a), who found that duration of courtship (in melanogaster) is determined by the female and duration of copulation by the male. Spieth (1952) states that species discrimination is a function of both sexes among closely related species and (1949) that both sexes can be responsible for courtship cessation (in species of the willistoni group). Spiess and Langer (1964) reported that the karyotype of each sex (in persimilis) influences mating speed.

The results of the chromosome assay not only verify the genetic basis of the traits selected for but also furnish

one of the few instances (for sexual isolation) in which the relative contributions of the chromosomes are known. Dobzhansky and Koller (1938) found that mating preferences in pseudoobscura and persimilis are determined by the autosomes. Bateman (1949) stated that pseudoobscura-persimilis hybrids display no chromosomally determined discrimination and that this is to be expected if discrimination is determined by a delicate gene balance which is destroyed in the hybrid. Ehrman also studied (1961) discrimination by using hybrids, in this case of various subspecies of paulistorum. In the hybrids of one pair of subspecies, sexual preference is determined primarily by genes located on chromosome II; in the hybrids of another pair, no one chromosome is more important than another in determining sexual preferences; in the hybrids of a third pair, the X chromosome had enough modifiers of sexual preference to neutralize the genes in the rest of the genome (but only X chromosome markers were available for this pair of subspecies).

Returning to the initial difficulty in obtaining matings in male selection series one, it will be recalled that when pairs of flies of the same age as in female selection were left together for the same length of time (four days), almost no matings occurred. This is certainly in accord with the various results which indicate that facilitation may be involved in small mass matings (Morgan, 1929; Barker, 1962b, 1967). Sturtevant (1915) found that the sexual success of

wingless males is increased when they are mixed with normal males. This is presumably because the wing vibrations of a male are not limited in their effect (of producing sound or wafting scent) to only the female being courted. Something of this sort may be responsible, in my experiments, for the greater than expected number of vials with more than one female inseminated. This is as though the presence of one mating facilitates another, although other possible explanations will be discussed (see Chapter VI, B). My failure to obtain any matings (male selection series 1) when one male was placed with two females is in agreement with Barker's finding (1967) (for melanogaster and simulans) that as the proportion of males rises, the percentage of females inseminated increases. Minor changes in the number or ratio of flies in a vial, however, seemed not to alter the frequency of insemination in these experiments (see Chapter VI, A).

The problem of obtaining matings when only one male was used (in order to know whether that one male had mated) was solved by using younger females (one to two days old) in pair matings. With this experimental design, some matings always occurred and selection could be made in the up direction. This is in agreement with Barker's (1962b) statement that mating between these two species more easily occurs before discrimination develops in the female. Greater success with younger females was also found by Pontecorvo (1942), Uphoff (1949), and Manning (1959b).

As in Manning's selection for fast and slow mating speed (1961), there were enormous fluctuations between generations of my selection experiments. These usually affected simultaneous lines in parallel fashion. Much of the fluctuation, however, was correlated with the seasonal effect, possible explanations for which will now be discussed. Three possible environmental variables are involved. The vials in which flies were stored (before mating) were kept in the laboratory at room temperature. Thermograph readings reveal that the temperatures in the laboratory were approximately the same in winter or summer (65 to 70° F.), but with a greater tendency to fall to the 60 to 65° range during the winter. Although the matings were all at the same temperature throughout the year, it is possible that these differences in storage temperatures could alter later mating behavior. Ehrman found (1966) that pseudoobscura males homozygous for the Arrowhead inversion sequence (Ar/Ar) are more successful in mating with Ar/Ar females if raised at 16° than if raised at 25° C., but this difference has to do with the pre-imaginal environment (which was always confined to the incubator in my experiments). Perhaps less relevant (because of the extreme conditions) is Kvelland's (1965) finding that a treatment of 0° C. for 30 minutes has no effect on mating activity of zero to two hour old males (melanogaster) but causes a reduction in mating activity of three day old males.

On the other hand, the light cues were also quite different in the two times of year. In the winter there was no light in the laboratory in the mornings until the relatively late sunrise or until someone turned on the light (8 AM or after), and the lights always remained on until late (midnight or 1 AM). In the summer early sunrises (and approximately the same time of turning off the lights) resulted in a relatively longer period of light each day. That this might affect behavior is shown by the way in which one second of light, delivered to a fly stock maintained in darkness many generations, will cause the re-establishment of a diurnal eclosion rhythm which favors greater eclosion during the cooler times of the day (Pittendrigh, 1954).

Finally, it is possible that seasonal changes in the earth's magnetic field configurations may influence behavior in such a way as to alter mating patterns. Picton (1966) observed the amount of turning of flies (melanogaster) from a straight line after their emergence from a narrow corridor into an expanded field and reported statistically significant responses to all the magnetic configurations employed.

There is almost no reference in the literature to seasonal differences in isolation. Mayr and Dobzhansky (1945) did find, however, that when persimilis males (from mixed cultures of persimilis and pseudoobscura) were placed with both persimilis and pseudoobscura females (also from mixed cultures) in a male choice situation, the isolation between

the two species in May and June was much higher than for flies from pure cultures but in July was a little lower than for flies from pure cultures. This decrease in isolation with the coming of summer is the opposite of my findings.

CHAPTER VI

OTHER EXPERIMENTS

A. Influence of Altered Sex Ratio on Mating Behavior

As mentioned in the Methods and Materials, I recorded the number of flies of each sex which died during a cross in order to study the possible effect upon hybridization of altering the number or sex ratio of the flies. In order to sample the possible effect of such alteration, I chose female selection series 1 for a detailed study. For each generation (and type of mating based upon time available for mating) in which all females were not recovered alive before the single female tests, I ran a contingency test with 2 x 2 tables (one degree of freedom) in order to compare the number of females inseminated in vials with all remaining alive with the number of females inseminated in vials in which one or more females had died. Out of 55 such comparisons, 26 in the down line and 29 in the up line, only three showed significant differences, two in the up line and one in the down line (this is a reasonable type I error for alpha = .05). The two comparisons with significant differences in the up line showed differences in opposite direction. Minor alterations in the sex ratio and the total number of females seem to have no effect on the success of hybridization.

For each generation (and type of mating based upon time available for mating) from the F-14 through the F-22 in which all males were not recovered before the single female tests, I made similar comparisons. Out of 48 such comparisons, 24 in each line, only one (in the up line) showed a significant difference (a reasonable type I error), and it was due to a higher than expected frequency of hybridization in the vials in which males had died. Since I (Chapter V, B) and others (Barker, 1967) have shown that decreasing the male to female ratio reduced the frequency of hybridization, it seems unlikely that altering the male to female ratio downward from 5:5 is responsible for increased hybridization in this one case, since 47 others showed no effect of the change.

B. Influence of one Mating on Another

It became apparent, with the use of single female tests, that inseminated females seemed not to occur at random among the five pair mating vials. Instead, a large number of inseminated females often came from the same few vials, even though a large number of vials had no inseminated females. In order to study this possible facilitation of one mating by another, I chose the mixed cultures experiment number six, both the control and the intrabottle experimental, for a more detailed statistical analysis because of the large numbers of five pair matings for which data were available. A frequency distribution for the numbers of vials with no,

one, two, three, four and five females inseminated was then compared with the expected frequency distribution based on a binomial distribution (see Appendix, 7). For both the control and the intrabottle experimental crosses, the differences were significant due mostly to the greater than expected number of vials with no females inseminated. The nature of the deviations was generally such as to indicate that there were more vials with no matings and similarly more vials with several matings than one would expect if the probabilities of mating are independent. Two possible reasons can be offered for this clumped distribution of inseminated females: (1) a more sexually active male inseminated several females in the same vial; or (2) one mating facilitated another. In the absence of direct observation of the mating of marked males in the five pair mating vials, there is no way to choose with certainty between these alternative hypotheses, and the results could be due to a combination of both factors, but (1) seems unlikely in light of the fact that out of a very large number of males (281) tested singly with two females in the F-1 generation of male selection series 1, none inseminated a female. On the basis of the two experiments above, at least some males should have been more sexually active. It is known, however, that in small mass matings, courting males may switch from one female to another instead of simply ceasing to court after ceasing to court a particular female (Manning, 1967b) and that females which are not even being courted may give

acceptance responses to stimuli from wing vibrations from nearby courting males (Spieth, 1952).

C. Influence of the Electric Field on Mating Behavior

During my other experimental work I had the opportunity (6-69) to place flies in an electric field in order to study its effect on mating behavior. With the apparatus available, vials could be placed on a wooden base and between the plates of a parallel plate aluminum capacitor; the plates were 10 cm. apart and 15 cm. high. I placed melanogaster females and simulans males (both from unselected stocks) together exactly as in all of the five pair crosses previously described for the major experiemnts and placed half the vials (experimentals) inside the electric field and half (controls) just outside the field. Both groups were at room temperature, but there was no way to regulate humidity. Females were placed singly into vials immediately upon removal from the field (in the case of the experimentals). The results are seen in Table XXIII.

Table XXIII

Results of Crosses in the Electric Field

treatment	no. of 5 pr.crosses	no. of females tested	% inseminated
control	10	50	22.0
30,000 v	10	49	10.2
control	6	30	10.0
15,000 v	6	30	20.0

The differences between controls and experimentals are not significant. Although the sample size is quite small, results indicate that the electric field does not have a striking effect upon mating behavior.

CHAPTER VII

GENERAL COMPARISONS

Since other workers also report such wide differences in success of hybridization (of melanogaster females with simulans males) as are found for the above experiments, it may be informative to briefly compare their results. Barker (1962b), using an experimental design similar to mine, set up pair matings at 25 ± 0.5° C. with nine to twelve hours of light per day; flies were discarded seven days later. Out of eight types of crosses involving wild type females and either mutant or wild type males, seven yielded no matings (out of 2406 pairs); one type of cross (involving mutant males and wild type females) yielded matings in 0.4 to 0.5% of the 434 pairs set up. When mutant females were placed with wild type or mutant males, 0.4% of the 483 pairs mated. This is similar to the low frequency of mating I obtained when using pair matings at the beginning of male selection series 1.

Barker (1967) again set up one week matings at 25 ± 0.5° C. and 65-70% relative humidity, with twelve hours of light per day. This time there were six pairs of flies per vial. Of five vials set up, none gave fertile cultures; of the fifteen females tested singly in vials (as a sample), none were inseminated. This, however, was an extremely small sample.

Uphoff (1949) placed ten three day old females and fifteen seven day old males into one ounce creamers at 26° C. for five days. From thirteen to 62% of the crosses yielded offspring in various experiments, with an average of 43% successful.

Biddle (1932) left four to five females with eight to ten males at 25° C. till pupae were formed. In some experiments all of the five or six such crosses set up were successful; in others, up to 50 unsuccessful attempts were made. The average success for all crosses was 44%.

Muller and Pontecorvo (1940) found that one-third of the crosses of triploid females with irradiated males were successful, and Pontecorvo (1943) routinely obtained nearly 100% fertile cultures by placing ten to twenty pairs of forked males and triploid females in 2 x 10 cm vials at 18 $\pm$ 2° C.

It should be noted that no dates were reported for the above melanogaster x simulans crosses. It is possible that some of the variation in results is due not only to difference in experimental design but also to seasonal influences similar to those reported here.

CHAPTER VIII

SUMMARY

1. Counter-conditioning young melanogaster females with simulans males did not alter the females' later sexual isolation from simulans males. However, experiments were performed at only one time of the year, and further experiments in other seasons would be needed for confirmation.
2. Rearing melanogaster and simulans together in the same culture bottles significantly reduced sexual isolation between the two species. There is an indication that additional generations of mixed culturing further reduces isolation.
3. That sexual isolation between the two species is partly genetic and influenced by both sexes is confirmed by successful selection, in both melanogaster females and simulans males, for increased and decreased isolation.
4. A chromosome assay for female selection indicates that all three major pairs of chromosomes contributed to the selected traits. Chromosome III contributed most to the difference between the up and down lines; chromosome II contributed least.
5. The results from many experiments over a period of two and one-half years indicate a cycle of increased isolation

between melanogaster females and simulans males in the summer and reduced isolation in the winter; possible reasons are discussed.

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APPENDIX

1. Test for independence of inseminations by "first" and "second" males in counter-conditioning experiment.

	no. of females inseminated by:				
	first males	second males	both	neither	total
obs.	24	98	1	389	512
exp.	20.2	94.2	4.8	392.8	512
dev.	3.8	3.8	-3.8	-3.8	

$X^2 = 3.91$ P = .01 - .05

2. Regression of transformed differences between up and down lines of female selection series 1 on number of generations of selection.

Source of variation	SS	df	MS	F_s
Linear Regression	1200.5	1	1200.5	19.99
Residual	1141.3	19	60.1	
Total	2341.8	20		

b = 1.25 $F_{.05(1,19)} = 4.38$

3. Regression of transformed differences between up and down lines of female selection series 1 on number of generations of selection:

   a. for the April-September interval.

Source of variation	SS	df	MS	F_s
Linear Regression	354.8	1	354.8	7.52
Residual	377.5	8	47.2	
Total	732.3	9		

b = 2.07 $F_{.05(1,8)} = 5.32$

b. for the October-March interval.

Source of variation	SS	df	MS	F_s
Linear Regression	906.6	1	906.6	13.42
Residual	607.8	9	67.5	
Total	1514.4	10		

b = 2.87 $F_{.05(1,9)} = 5.12$

When the two slopes are compared, $F_s = .10$; $F_{.05(1,17)} = 4.5$.

4. Regression of transformed differences between up and down lines of female selection series 2 on number of generations of selection.

Source of variation	SS	df	MS	F_s
Linear regression	47.6	1	47.6	1.18
Residual	403.0	10	40.3	
Total	450.6	11		

b = .58 $F_{.05(1,10)} = 4.96$

5. Regression of transformed differences between up and down lines of male selection series 2 on number of generations of selection.

Source of variation	SS	df	MS	F_s
Linear Regression	839.5	1	839.5	5.39
Residual	623.4	4	155.9	
Total	1462.9	5		

b = 6.93 $F_{.05(1,4)} = 7.71$

6. When the slopes for the two female selection lines (series 1 and 2) are compared, $F_s = .27$; $F_{.05(1,29)} = 4.18$.

7. Comparison of the numbers of vials with 0, 1, 2, 3, 4, and 5 matings with the expected frequency according to a binomial distribution.

a. control

no. inseminated	expected		observed		deviation	dev^2 / exp
5	.0194		0		-	
4	.4850	5.9364	1	5	+	.15
3	5.4320		4		-	
2	29.2940		9		-	14.06
1	77.2508		17		-	46.99
0	81.1114		163		+	82.67
total	193.5926		194		X^2 = 143.87	
					P = less than .05	

b. control

no. inseminated	expected		observed		deviation	dev^2 / exp
5	.2338		1		+	
4	3.1563	20.8249	5	15	+	1.63
3	17.4348		9		-	
2	47.3445		15		-	22.10
1	63.9944		15		-	37.51
0	34.6024		122		+	220.75
total	166.7662		167		X^2 = 281.99	
					P = less than .05	