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# Developmental isolation and subsequent adult behavior of *Drosophila paulistorum*

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City University of New York, 1994

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# Developmental Isolation and Subsequent Adult Behavior of *Drosophila paulistorum*

by

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# ABSTRACT

## Developmental Isolation and Subsequent Adult Behavior of *Drosophila paulistorum*

by

Yong-Kyu Kim

Advisor: Professor H. Roberta Koepfer

The six *Drosophila paulistorum* semispecies were tested for sexual isolation before and after the following six treatments: Seclusion, total physical isolation from consemispecifics; long term chemical and vibratory contact (LTCVC), exposure to airborne heterosemispecific stimuli from egg through sexual maturity; postimaginal contact (PIC), physical contact with heterosemispecifics during the postimaginal period only; selection, total physical contact with heterosemispecific individuals during preimaginal stages; relaxation, pure culturing without total physical contact; reselection, reinstatement of selection in two ways, LTCVC and mixed culturing: ↑ = isolation significantly increased; - = isolation was not changed significantly; and ↓ = isolation significantly decreased.

| TREATMENT | SEXUAL ISOLATION |
|---|---|
| Seclusion | ↓ |
| LTCVC | ↑ |
| PIC | – |
| Selection | ↑ |
| Relaxation | ↓ |
| Reselection | ↑ |

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## Table of Contents

## List of Tables

## List of Illustrations

# INTRODUCTION

Mate choice may be defined as any behavior pattern, displayed by members of one sex, that leads to the greater likelihood of their mating with certain members of the opposite sex (Halliday, 1983). When choosing a mate, an animal utilizes various estimates of potential mate quality in terms of either short term benefits or long term benefits or both. Choosing a mate with high quality increases an animal's adaptive fitness and so such a behavior should be favored by natural selection (Halliday, 1978; Hamilton and Zuk, 1982; Trivers, 1972; Zahavi, 1975). Further, the choice of a conspecific mate is adaptive in that it prevents production of relatively unfit hybrids. An individual organism, however, has various experiences during its development to adult sexual maturity and previous experiences may influence subsequent behaviors, including mate choice. Many studies have dealt with the influences of early experiences on the development of the mating preferences.

Generally, the distribution of sexual behaviors among male and female primates is strongly influenced by the hierarchies under which these behaviors take place (Cowlishaw and Dunbar, 1991; Keverne, 1992). Primates reared in various restricted conditions, however, showed delayed and distorted development of sexual behaviors and mating preference varied with rearing conditions (see review in Harlow and Harlow, 1969; Hanby, 1976). Similar findings have been reported for other species reared in isolation; for junglefowl (Kruijt, 1964, 1971); for the black-headed gull (Groothuis and Mulekom, 1991); for the domestic cat (Baerends van Room and Baerends, 1979); and for several fish species (Tooker and Miller, 1980).

Preferences acquired during early development can be changed by differences in rearing conditions. In zebra finches, it has been shown that early experiences with fostering parents influence mating preferences via sexual imprinting on the parents, but such imprinted preferences can be modified by later experiences (Clayton, 1990a, b & c; Immelmann et al., 1991; Kruijt and Meeuwissen, 1991). Sexual preference for a previously-experienced color morph has been reported in cichlid fishes (Baerends and Baerends van Roon, 1950; Noakes and Barlow, 1973; Sjölander and Fernö, 1973; Fernö and Sjölander, 1976; Weber and Weber, 1976).

There are many studies involving the effects of previous experiences on Drosophila mating behaviors (see review in Siegel et al., 1984; Spieth and Ringo, 1983). When sexually mature males and females are brought together, males display courtship and females respond to the normal courtship, which is followed by successful matings (see Ewing, 1977). Mature males also come into contact with immature males and females, and often attempt to court unsuitable partners such as immature males, which emit female-type odor when they are very young (Cook and Cook, 1975; Jallon and Hotta, 1979). D.

melanogaster males which have had that experience show reduced levels of courtship toward immature males upon a second exposure (Gailey et al., 1982; Zawistowki and Richmond, 1985, 1987) and therefore, they are more successful than naive males at mating virgin females. On the other hand, fertilized females display rejecting behaviors toward courting males (Connolly and Cook, 1973) and males paired with fertilized females show a depressed courtship activity with virgin females in subsequent mate choices (Siegel and Hall, 1979; Tompkins and Hall, 1981). This reduction of courtship is associated with the release of chemical compounds from fertilized females in response to courting males (Gailey et al., 1984; Gailey and Siegel, 1989; Tompkins et al., 1983). In D. silvestris, however, females mate equally with depleted males and recovered males (Schwartz, 1991).

In addition, Ehrman and collegues have demonstrated that age, previous copulatory experience, and exposure to other males and females can alter the succeeding mate choice of female D. pseudoobscura (Ehrman, 1989, 1990; Pruzan, 1976; Pruzan and Ehrman, 1974); and that D. paulistorum females, previously mated homogamically, are more likely to repeat this experience in subsequent matings than are those which have mated heterogamically (O'Hara et al., 1976; Pruzan et al., 1979). While male D. subobscura kept in isolation are more successful in mating than those reared in groups (Maynard Smith, 1956), D. silvestris, as a consequence of social isolation, exhibit decreased frequency of success (Sene, 1977). However, relatively little is known about the effect of early experience on sexual isolation, defined as "the weakness or absence of behavioral attraction between species" (Dobzhansky, 1970).

Sexual isolation may also be a byproduct of adaptive forces shaping other behaviors in local demes (Paterson, 1978, 1982; Carson and Lande, 1984; Carson, 1987). A few studies reported that sexual isolation could be influenced by different types of culture media in the laboratory or in nature (Brazner and Etges, 1992; Etges, 1992; Wasserman and Zweig, 1991, but see Markow, 1991). What then are the effects of experience on sexual isolation between closely related species? What are the effects of preadult contact with other individuals or lack of such contact at early stages in the life cycle? Is sexual isolation influenced by preadult experience?

Here, a series of experiments focusing on the development of discriminatory behavior over the entire lifetime of holometabolous D. paulistorum will be described. I examined the effects on mate choice of total physical isolation at each of six developmental stages, e.g., egg, first-, second-, and third-instar larvae, pupa and adult, and investigated the consequences of exposure both to consemispecifics and to heterosemispecifics during each stage. Conversely, I have comprehensively investigated mate choice effects mediated by lifelong and/or limited contact with intersterile semispecies.

## Drosophila paulistorum superspecies complex

Drosophila paulistorum Dobzhansky and Pavan (Burla et al., 1949) is a neotropical species. It comprises six incipient species (Dobzhansky and Spassky, 1959). These are called the Centroamerican, Orinocan, Amazonian, Andean-South Brazilian, Interior, and Transitional semispecies. They are mostly allopatric, but in some places their geographic ranges overlap, and two or more semispecies may coexist sympatrically without interbreeding (Figure 1a). The six semispecies have almost reached the status of reproductively isolated, but morphologically indistinguishable, sibling species. However, with the exception of the Transitional semispecies, which shares chromosomal polymorphisms with the Andean-South Brazilian one, each semispecies maintains its own exclusive inversion polymorphisms (Dobzhansky and Pavlovsky, 1962; Kastritsis, 1966, 1967, 1969). Figure 1b represents the phylogenetic relationships among the semispecies as indicated by the available data on cytology, morphology, behavior and ecology (Spassky et al., 1971). Centroamerican, Transitional and Andean-South Brazilian semispecies are closely related to each other. The Interior semispecies is closely related to Orinocan, and the Amazonian semispecies is the most distinct of the six semispecies. Further, electrophoretic analysis shows that the Centroamerican semispecies is more similar to the Transitional one than to the Andean-South Brazilian one (Figure 1c).

Two reproductive isolating mechanisms, sexual isolation and hybrid sterility, are involved in the D. paulistorum speciation process (Ehrman and Powell, 1982). Many degrees of sexual isolation exist within this species complex, and sexual isolation varies from semispecies to semispecies and from strain to strain. By and large, when geographically different strains of the same semispecies are tested together in sexual isolation experiments, homogamic matings are more frequent than heterogamic ones. Intersemispecific crosses show female preferences for homogamic males (Carmody et al., 1962; Ehrman and Wasserman, 1987). For given pairs of semispecies, collected in nature both sympatrically and allopatrically, pairs occurring sympatrically exhibit stronger sexual isolation than do pairs occurring allopatrically (Dobzhansky et al., 1964; Ehrman, 1965). Ehrman (1961) has shown that the genetic basis of the sexual isolation in D. paulistorum is polygenic and distributed on all the three chromosome pairs possessed by this species complex.

No intersemispecific hybrids have ever been found in nature. Where two or more semispecies coexist sympatrically, they apparently do not interbreed, thus behaving like full-fledged species (Ehrman, 1965). Hybrid progeny resulting from intersemispecific matings, under laboratory conditions, consist of fertile females and sterile males (Dobzhansky and

Spassky, 1959). Further, fertile females and sterile males are produced for at least seven generations when the fertile females are backcrossed to one of either parental males. The hybrid sterility is due to incompatibilities between maternally transmitted endosymbionts (cell-wall-deficient bacteria or L-forms) and paternal genetic material (Ehrman, 1960a & b, 1961; Ehrman et al., 1986; Ehrman and Kernaghan, 1971; Somerson et al., 1984a & b; Williamson and Ehrman, 1967, 1971; Williamson et al., 1971). The presence of a foreign endosymbiont in a hybrid male results in rapid proliferation of these L-forms in the testes of that male and the sterility is inherited and transmissible. However, individual strains representing the Transitional semispecies, from which the other five semispecies have presumably descended, can be crossed and produce fertile hybrids with some of the other five semispecies. Gene flow between the incipient species is therefore possible, and D. paulistorum is regarded as a single species (Dobzhansky and Spassky, 1959; Dobzhansky et al., 1964, 1969; Spassky et al., 1971). The fact that sexual isolation and hybrid sterility in D. paulistorm were weakly and incompletely correlated (Carmody et al., 1962; Dobzhansky et al., 1969) caused Dobzhansky to hypothesize that the D. paulistorum hybrid sterility constituted the primary isolating mechanism, and that the sexual isolation developed later by natural selection, in response to the production of consistently unfit hybrids (Ehrman, 1962; Dobzhansky and

Pavlovsky, 1967; Williamson and Ehrman, 1967). This contradicts Mayr's (1988) view, which parallels that of Muller (1942), that sexual isolation arises as a byproduct of adaptation to different geographic areas. Thus, this complex provides opportunities for studying the processes underlying speciation, mating choice behavior, and the relationship between an insect host and its microbial symbiont.

# EXPERIMENTAL DESIGN AND METHODS

## Materials

The geographic strains and semispecies utilized here originated from:

1) Lancetilla, Honduras: Centroamerican (CA)
1a) Georgetown, Guyana: Orinocan (OR)
2) Mesitas, Colombia: Andean-South Brazilian (AN)
2a) Santa Marta, Colombia: Transitional (TR)
3) Belem, Brazil: Amazonian (AM)
3a) Llanos, Colombia: Interior (IN)

The geographic location of these six allopatric strains is shown in Figure 2 and Figure 3 shows the relative degrees of sexual isolation among them. The Amazonian semispecies is the most distinct of all, and is strongly isolated from each of the others except Transitional. In contrast, Transitional generally shows partial isolation in all combinations, and thus is the semispecies which would enable gene flow within the superspecies. The Santa Marta strain of Transitional, however, does produce sterile males with strains of the Andean-South Brazilian semispecies (Ehrman, 1963a & b; Williamson and Ehrman, 1967). See Set 2 above. The Interior semispecies is partially isolated from all but the Amazonian, from whom it is strongly isolated (See Set 3 above). It crosses most easily with the Orinocan semispecies, and has never been found in sympatry with that semispecies although their distribution areas are geographically adjacent (Dobzhansky et al., 1969; Perez-Salas et al., 1970; Dobzhansky and Pavlovsky, 1975). Thus, reproductive isolation between the Interior, Amazonian and Andean-South Brazilian semispecies is secure enough to make the sympatry possible with little or no gene exchange.

These semispecies were surveyed in pairs in each of three different experiments; that is, effects of developmental isolation, long term chemical and vibratory contact, and postimaginal contact on D. paulistorum mating behaviors were investigated as explained below.

## Methods

Most studies of sexual isolation in Drosophila utilize young virgin flies that have been reared communally with homogamics, and aged with homogamics of the same sex. Few studies have excluded the possible effects of exposure to other flies by rearing individuals in total isolation from conspecifics (Ehrman, 1990; Koepfer and Fenster, 1991), or have addressed the issue of mixed culturing, i.e., exposure of two or more types to each other during the rearing process (Eoff, 1973; Kessler, 1966; Koopman, 1950; Manning, 1959; Mayr and Dobzhansky, 1945), a protocol that mimics to some extent the situation often found in feeding/breeding areas in nature.

I have employed both of these approaches. Flies isolated at various developmental stages are henceforth referred to as "secluded" flies to avoid confusion with the term "isolated," as used in the context of sexual isolation.

1) DEVELOPMENTAL ISOLATION (SECLUSION) STUDY: Flies were secluded at each of the six developmental stages, giving seven types from each of six semispecies as summarized in Table I, i.e., six kinds of secluded experimentals (S1 through S6) and one control (CT).

For egg seclusion, microscope slides were coated with a thin layer of blue Drosophila medium. Single slides were placed inside half-pint bottles; gravid females were then added and allowed to oviposit for 24 hours. Individual eggs were removed with a toothpick and placed on the surface of the medium in a small glass creamer containing a strip of moist toweling. This creamer was capped and set aside until the imago emerged. Imagoes (S1) were stored alone for three days to sexual maturity, at which time they were tested. A very low hatchability (approximately 30%) was observed in S1.

These steps were repeated for first-, second-, and third-instar larvae. First instars were collected from slides held for 24 hours after the 24 hours oviposition period, second instars were collected from slides held for 48 hours after the oviposition period, and third instars were collected from slides held for 96 hours after the initial 24 hours interval.

Visual differences in larval mouthparts expedited the determination of larval age. Emerging imagoes were types S2, S3, and S4.

For pupal seclusion, gravid females were placed into half-pint bottles and allowed to oviposit for 24 hours. After removal of these females, moist strips of paper toweling were added. Pupae were subsequently collected from the toweling and placed singly in creamers, giving rise to S5 imagoes. Flies secluded as imagoes were raised in mass cultures in bottles. At the moment of emergence, these S6 individuals were placed into creamers and aged.

Controls were raised in mass cultures, collected upon emergence, and aged communally.

<u>2) LONG TERM CHEMICAL AND VIBRATORY CONTACT (LTCVC):</u> In these experiments, members of two semispecies were subjected to chemical and vibratory contact with each other during a whole life cycle. The semispecific combinations utilized in the seclusion experiments were also used here. For each combination, five mass cultures of each semispecies were initiated with 50 pairs of adult virgins per half-pint culture bottle. Five pairs of heterosemispecific LTCVC cultures were then created, each pair consisting of two culture bottles connected by a glass bridge (Figure 4). The bridge was divided into two parts by a fritted glass barrier that prevented movement of flies between the cultures, but allowed vibratory

stimulation and passage of airborne chemicals between the semispecies. These LTCVC cultures were maintained to Generation 10 in order to provide data on differences between generations. Each generation, virgin flies, collected from the five bottles of each semispecies, were aged for three days, and then used for mating choice observations. Five new mass cultures of each semispecies were established with 50 pairs of adult virgins per bottle as before, after collecting and mixing virgins from all the five bottles in an attempt to prevent inbreeding and/or any drift effect on mating behavior (Powell, 1978; Powell and Morton, 1979; Ringo et al., 1986, 1987).

3) POSTIMAGINAL CONTACT (PIC): To determine whether sexual preference acquired from conspecifics during a sensitive period is relatively stable (Immelman, 1972; Immelman and Suomi, 1981; Lorenz, 1935, 1982), individuals of a semispecies were subjected to a relatively short exposure to heterosemispecifics with physical contact: Newly emerged virgin flies, all of one semispecies and gender, were placed with mass reared adult flies of another semispecies but the same gender, after which the two types were left together for three days in a ratio of 50:50 in a half-pint bottle. As shown in Table VII, PIC was surveyed in seven different ways. The experimental flies were separated after PIC treatment and then given mate choices representing each of the seven PIC

combinations. The null hypothesis was that there would be no significant differences between communally raised flies and experimentals. The same semispecies paired in the seclusion experiments were tested via postimaginal contact.

4) SELECTION WITH MIXED CULTURING, RELAXATION, AND RESELECTION: To test the mixed culturing effect on mating behaviors, through complete physical contact with heterosemispecifics during the entire developmental process, I utilized mutant strains of two different semispecies, that is white-eyed Centroamerican and yellow-bodied Andean-South Brazilian, because wildtypes are morphologically indistinguishable and the number of mutant strains is limited in D. paulistorum (see Appendix 1). There were five lines: Control (CT), Selected (S), Relaxed (R), Reselected-1 (RS-1), and Reselected-2 (RS-2). Controls were reared in mass culture, sexed, and aged communally. For the Selected line, flies were reared via mixed culturing, utilizing the following procedures: Gravid females from the Mesitas, Colombia (AN) and Lancetilla, Honduras (CA) strains were placed together into culture bottles and allowed to oviposit for 24 hours, after which they were removed. Virgins of both strains were collected from cultures and allowed to mate and produce progeny in culture bottles. Homogamic matings yield offspring that are phenotypically mutants; heterogamic matings give wildtype offspring. Wildtype individuals were discarded, and

the mutant flies were allowed to become parents of the next generation. This imposes a selective advantage on genetic constitutions which favor matings within, and discriminate against, matings between strains. The Selected line was maintained in five culture bottles, each of which was initiated each generation with approximately 25 pairs from each of the two strains.

After 14 generations of mixed culturing, the Relaxed line was established. Approximately 200 pairs of each semispecies were used as founders of the CA and AN Relaxed lines, each composed of five culture bottles. After five generations under relaxed conditions, once again 200 pairs of each semispecies from the Relaxed line were subjected to renewed selection via mixed culturing, which was termed Reselected-1, and another 200 pairs were subjected to selection via chemical and vibratory contact with glass bridges, which was called Reselected-2. Both Reselected-1 and Reselected-2 lines were composed of five culture bottles each. Every generation all virgin flies including the Control line were routinely mixed within lines, and were aged for three days before testing for mate choices.

## BEHAVIORAL TESTING

### Female Choice

Three-day-old flies from the seclusion experiment were introduced into Elens-Wattiaux chambers (Elens and Wattiaux, 1964) without anesthesia, and observed in forty-two combinations for each of the three pairs of semispecies (Table II, III and IV). For each combination, the chamber contained twenty-five females, twelve homogamic males and twelve heterogamic males. There were four replicates per combination; in two replicates, the left wing of the homogamic male was notched and in the others the left wing of the heterogamic male was notched. In this superspecies, it has been demonstrated that clipping does not affect mating propensity or discrimination (Petit and Ehrman, 1969). Observations continued until 25 matings had occurred, which occurred within an hour. Throughout mating observations, females mated only once during the duration of the tests and copulations lasted approximately 15-20 minutes.

### Multiple Choice

This method was used for behavioral observations of flies from the seclusion experiment (Table V) and the other three experimental regimes (Table VI, VII and VIII). For these tests, twelve females and twelve males of each of two strains (48 flies in all) were placed in each chamber with five replicates. Flies of one of the two strains had a part of one wing clipped to distinguish morphologically similar strains. Observation continued until 120 matings had occurred.

## Statistical Analysis

The Isolation Index, I (Malogolowkin-Cohen et al., 1965), was used to determine the degree of sexual isolation exhibited in multiple choice combination. In the formulas below, $x_{AA}$, $x_{BB}$, $x_{AB}$ and $x_{BA}$ are the number of matings between females and males of strain A, females and males of strain B, females of strain A and males of strain B, and females of strain B and males of strain A, respectively, and N = the total number of matings.

$$I = \frac{x_{AA} + x_{BB} - x_{AB} - x_{BA}}{N}$$

I ranges from 1 (total isolation, all homogamic matings) through 0 (random mating) through -1 (all heterogamic matings). The standard error (SE) of this index is $[(1-I^2)/N]^{1/2}$.

For female choice combinations, the formula for I is modified as follows, but that for SE is unchanged.

$$I = \frac{x_{AA} - x_{AB}}{x_{AA} + x_{AB}}$$

According to the null hypothesis, it is assumed that control and experimental mating patterns would not differ significantly regardless of experimental rearing method. One-way ANOVA tests were performed with raw data using the F test to examine the variation of means across replicates for each mating combination (Sokal and Rohlf, 1981). The T-method was used for comparisons of potentially significant differences among all possible pairs of means. Regression analysis was conducted to look at the relationship between two parameters; homogeneity of regression coefficients was tested. For these statistical analyses, however, transformation was inappropriate in this study because the isolation index ranges from -1 to +1. See Appendix 2 for further information.

## RESULTS

### Seclusion Experiments

Table II, III and IV show the results of reciprocal female choice tests involving Centroamerican with Orinocan, Andean-South Brazilian with Transitional, and Amazonian with Interior, respectively. The forty-two different combinations for each pair are divided into Sets A and B by female semispecies, and into Subsets A1, A2, A3, B1, B2, and B3 by seclusion treatment. In the A1 and B1 tests, only the females were secluded and all males came from control lines. In A2 and B2, only the homogamic males were secluded. In A3 and B3, both females and homogamic males had been subjected to seclusion.

### 1) Intersemispecific Tests: Centroamerican (CA) x Orinocan (OR)

Chi-square one-sample tests were used to examine each combination for departure from random matings; the null hypothesis was equality of homogamic and heterogamic matings (Ringo et al., 1986). Most combinations exhibited highly significant excesses of homogamic matings ($p \leq 0.001$), and highly significant isolation indices. However, three combinations in Subset A2, two in A3 and four in B2 displayed random mating after seclusion.

Subsets A1, A2 and A3 are tests involving CA♀♀ x (CA♂♂ + OR♂♂) semispecies, for which the controls show a relatively high degree of sexual isolation (84% homogamic matings, I = 0.68±0.07). There were no significant differences within Subset A1 ($F_{6,21} = 0.93$, $p>0.05$). There were highly significant differences within Subsets A2 and A3 respectively, however ($F_{6,21} = 5.17$, $p\leq0.01$; $F_{6,21} = 4.68$, $p\leq0.01$). In A2, behavior of the control lines was highly significantly different from that of the secluded experimentals ($F_{1,21} = 10.70$, $p\leq0.01$). There were also significant differences within the A2 secluded group ($F_{5,21} = 4.07$, $p\leq0.01$), and three classes, S1 - S3, of secluded females exhibited significantly decreased isolation when compared to their controls. Subset A3 also exhibited significant differences between the control lines and the secluded experimentals ($F_{1,21} = 8.57$, $p\leq0.01$). So did within secluded group test ($F_{5,21} = 3.91$, $p\leq0.05$); and isolation decreased significantly in two combinations, S1 and S2.

Subsets B1, B2 and B3 show the reciprocal test results, OR♀♀ x (OR♂♂ + CA♂♂), for which the controls show a relatively low degree of sexual isolation (I = 0.40±0.09). There were no significant differences within Subset B1, B2 and B3 respectively ($F_{6,21} = 0.69$, $p>0.05$; $F_{6,21} = 2.35$, $p>0.05$; $F_{6,21} = 1.08$, $p>0.05$). Sexual isolation did not change significantly in any combination here ($p>0.05$).

Relationships between secluded stage and isolation index were tested: all but Subset B2 showed the presence of significant linear regression (Figure 5). The analysis therefore suggests that stages in which seclusion is initiated early have low isolation indices. Homogeneity among regression coefficients of the three different seclusion combinations from each of CA and OR was tested. CA did not differ in response to seclusion depending on seclusion combinations ($F_{2,15}$ = 3.67, $p>0.05$); there was a significant difference between Subsets A1 and A2, however ($p \leq 0.05$). On the other hand, three regression coefficients were homogeneous in OR ($F_{2,15}$ = 0.27, ns); that is, no differences were recorded in responses to seclusion regardless of sex.

2) Intersemispecific tests: Andean-South Brazilian (AN) x Transitional (TR)

Chi-square one-sample tests showed that all combinations in Table III, except S1 and S2 in both Subsets B2 and B3, had highly significant excesses of homogamic matings ($p \leq 0.001$).

Subsets A1, A2 and A3 are tests involving AN♀♀ x (AN♂♂ + TR♂♂), for which the control flies show an almost perfect degree of isolation (98% homogamic matings, I = 0.96±0.03). There were significant differences within Subset A1, A2 and A3, respectively ($F_{6,21}$ =3.79, $p \leq 0.05$; $F_{6,21}$ = 5.60, $p \leq 0.01$; $F_{6,21}$ = 4.13, $p \leq 0.01$). In A1, there were significant differences between the control and the secluded experimentals, and also within the secluded group ($F_{1,21}$ = 4.82, $p \leq 0.05$; $F_{5,21}$ = 3.58, $p \leq 0.05$). Sexual isolation significantly decreased only in the combination S1. In A2 and A3, respectively, there were also significant differences between the control line and the experimentals ($F_{1,21} = 7.44$, $p \leq 0.05$; $F_{1,21} = 5.55$, $p \leq 0.05$), and also within the secluded group ($F_{5,21} = 5.23$, $p \leq 0.01$; $F_{5,21} = 3.85$, $p \leq 0.05$). In combinations S1 and S2, isolation was significantly decreased.

In Subsets B1, B2 and B3 of reciprocal tests involving TR♀♀ x (TR♂♂ + AN♂♂) semispecies, however, a relatively low degree of sexual isolation was recorded in the control line (I=0.54±0.08) when compared to that of the reciprocal. There were no significant differences within each of the Subsets ($F_{6,21} = 0.08$; $F_{6,21} = 0.41$; $F_{6,21} = 0.37$).

In Subset B1, linear regression became non-significant ($t_s = 1.539$, $p > 0.05$). All other Subsets showed significant regression relationships between secluded stage and isolation index, however (Figure 6). There was no significant difference among three seclusion combinations in response to seclusion in both AN and TR ($F_{2,15} = 0.01$; $F_{2,15} = 2.92$).

### 3) Intersemispecific Tests: Amazonian (AM) x Interior (IN)

Table IV shows the results of reciprocal tests between AM and IN. Each combination exhibited a large number of homogamic matings ($p \leq 0.001$), and a highly significant isolation index.

In Subsets A1, A2 and A3, the controls show a very high degree, almost complete, of sexual isolation (98% homogamic matings, I=0.96±0.03). In A1, behavior of the control lines were highly significantly different from that of the experimentals ($F_{1,21} = 23.67$, $p \leq 0.001$). There were also significant differences within the secluded group ($F_{5,21}$ = 6.08, $p \leq 0.01$); secluded females, S1 and S2, exhibited isolation significantly lower than the controls ($p \leq 0.01$). There were significant differences within Subset A2 ($F_{6,21}$ = 6.13, $p \leq 0.001$), within the secluded A2 group ($F_{5,21}$ = 4.48, $p \leq 0.01$), and between the control line and the secluded experimentals ($F_{1,21}$ = 14.40, $p \leq 0.001$). Sexual isolation decreased significantly in combinations S1 and S2. There was also significant heterogeneity within Subset A3 ($F_{6,21}$ = 3.47, $p \leq 0.05$), significant differences within the secluded group ($F_{5,21}$ = 2.82, $p \leq 0.05$), and between the control line and the secluded experimentals ($F_{1,21}$ = 6.74, $p \leq 0.05$): Combination S1 exhibited significantly decreased isolation ($p \leq 0.05$).

The reciprocal tests, Subsets B1 - B3, also exhibited a nearly complete sexual isolation in the control lines (99% homogamic matings, I=0.98±0.02). There were highly significant differences within Subset B1 ($F_{6,21}$ = 4.26, $p \leq 0.01$), and the isolation exhibited by S1 females was significantly lower than that shown by controls (I=0.58±0.08). However, there were no significant differences within Subsets B2 and B3 ($F_{6,21}$ = 0.85; $F_{6,21}$ = 1.02).

All but Subset B1 showed the presence of significant linear regression relationships between secluded stage and isolation index (Figure 7). Three regression coefficients in AM and IN respectively were not heterogeneous; there was no difference in response to seclusion between sex(es) involved in seclusion ($F_{2,15}$ = 1.13; $F_{2,15}$ = 2.82).

### 4) Intrasemispecific Matings:

Table V shows the results of the seclusion effect on intrasemispecific D. paulistorum matings. For these tests, twelve pairs of communally raised controls (C) and twelve pairs of secluded experimentals (S1-S6), within a given semispecies, were observed per chamber, from which three isolation indices were calculated; that is I (Joint Isolation Index), Ic and Is (Female Isolation Index). There were no significant differences within any of the Subsets ($F_{5,24}$ = 1.08; $F_{5,24}$ = 1.67; $F_{5,24}$ = 0.53; $F_{5,24}$ = 0.98; $F_{5,24}$ = 0.38; $F_{5,24}$ = 1.57); isolation indices fluctuated without any definite trend, i.e., they gradually increased and/or decreased, regardless of the stages at which individuals were secluded. 22 out of 36 Joint Isolation Indices (I) show significant deviations from random mating, however. In Female Isolation Indices (Ic and Is), 19/36 and 13/36 indices significantly deviated from zero (random mating).

## **Long Term Chemical and Vibratory Contact**

Members of each of three pairs of semispecies, CA and OR, AN and TR, and AM and IN, were exposed to each other via a fritted glass bridge, permitting access to airborne chemical and vibratory stimulation (Table VI). There were no significant differences within Set A ($F_{6,28}$ = 2.16, ns). The isolation index increased one generation after the two semispecies were LTCVC treated, but it was not significantly different from the degree of sexual isolation shown by the control line. At Generation 10, however, the experimental lines exhibited significant isolation from the control line ($p \leq 0.05$); complete isolation index ($I = 1.00$) has maintained since then.

In Sets B and C, the degrees of sexual isolation between the control lines were almost perfect ($I = 0.97 \pm 0.02$). In Set B, isolation indices fluctuated profoundly during the initial generations, but leveled off around the $I = 0.70$ level. None of the experimental lines were significantly different from the control lines ($F_{6,28} = 2.02$, ns). Isolation indices were not significantly different from those of the controls in Set C. The indices, however, have remained at 1.00 since Generation 4; there were significant differences within the Set ($F_{6,28} = 3.76$, $p \leq 0.01$).

**Postimaginal Contact**

Table VII shows data from the seven PIC combinations for each of the three pairs which were used in conjunction with multiple choice testing. In Set A, all combinations, except those in which only CA♂♂ were subjected to PIC, showed lower isolation indices than that of the control lines, and there were significant differences among the seven types of experimentals and controls ($F_{7,32} = 2.43$, $p \leq 0.05$).

In Set B, when AN was subjected to PIC in three combinations, there were no significant differences between the experimentals and the controls ($p>0.05$). Three types of treatment for TR significantly lowered isolation indices, however ($p\leq0.01$). When the two semispecies were exposed to each other, their mating discrimination was not influenced significantly ($p>0.05$). In Set C, the experimental combinations showed no significant differences from the controls ($F_{7,32} = 1.80$, ns).

## Selection with Mixed Culturing, Relaxation and Reselection

### 1) Selection with Mixed Culturing

Table VIII and Figure 8 show the D. paulistorum mating response to several rearing methods. When CA and AN were reared together, the isolation index rose significantly from 0.57±0.08 to 0.90±0.04 after one generation of mixed culturing ($F_{1,8} = 11.69$, $p\leq0.01$), and it remained at this level for the subsequent generations of mixed culturing (Set B, Table VIII). This was so because numbers of heterogamic matings between CA females and AN males decreased, and numbers of homogamic CA matings increased. At Generation 0, there were no matings between AN females and CA males, but 26 matings between CA females and AN males. By Generation 22, the number of such heterogamic matings dropped to six out of sixty matings (5%). There were no significant differences in homogamic AN matings during this period of mixed culturing.

At Generation 43 of mixed culturing, sexual isolation between Control (parent) and Selected (daughter) lines within the same semispecies was tested to see whether behavioral changes affecting intrasemispecific mate choices had occurred in the Selected line after about four year of mixed culturing (Set F & G, Table VIII). There was highly significant isolation between Control and Selected lines in each of two semispecies ($I_{CA}$ = 0.68±0.08; $I_{AN}$ = 0.62±0.08). There was no asymmetric isolation between AN (Ic = 0.53±0.11; Is = 0.70±0.09; $F_{1,8}$ = 0.93, ns), but there was strong asymmetric isolation between the CA lines (Ic = 0.93±0.05; Is = 0.43±0.12; $F_{1,8}$ = 6.82, $p \leq 0.05$).

## 2) Relaxation

When mixed culturing was relaxed at Generation 14, and these two semispecies were cultured separately, numbers of heterogamic matings between two semispecies began to increase; relaxation not only resulted in a renewal of CA♀♀ x AN♂♂ matings, but also in an increased frequency of the reciprocal heterogamic matings, AN♀♀ x CA♂♂. The isolation index dropped significantly from 0.97±0.02 at Generation 14 to 0.48±0.08 at Generation 17 ($F_{1,8}$ = 31.61, $p \leq 0.001$); it has remained at levels that are significantly below the control level for the remaining five generations (Set C, Table VIII).

### 3) Reselection

The Relaxed lines were reselected at Generation 19 in two different ways: mixed culturing; and chemical and vibratory contact, as in LTCVC. Both mixed culturing (Set D, Table VIII) and LTCVC (Set E, Table VIII) resulted in significantly increased isolation. At Generation 22, isolation between the RS-1 lines (mixed culturing) was significantly higher than that between the Relaxed lines ($p \leq 0.05$); and the RS-2 lines also differed significantly from the Relaxed lines ($p \leq 0.01$). The three disparate selection lines, Selected, Reselected-1 and Reselected-2, have converged to about the same isolation index value, that of I = 0.90 ($F_{2,20}$ = 0.14, ns). Control lines have been maintained throughout the course of this experiment, and their isolation indices have not changed significantly (Set A, Table VIII).

## DISCUSSION

My results, representing a total of 27,360 observed matings, may be tallied according to semispecies, and according to rearing treatment, as follows:

A. the Drosophila paulistorum semispecies
   1) Centroamerican x Orinocan
   2) Andean-South Brazilian x Transitional
   3) Amazonian x Interior
   4) Andean-South Brazilian x Centroamerican

B. alternative methods of rearing
   1) developmental isolation (seclusion)
   2) long term chemical and vibratory contact
   3) postimaginal contact
   4) mixed culturing

Several studies have reported the effect of isolation after eclosion on mating behavior of Drosophila. Male and female D. melanogaster subjected to isolation, after eclosion, showed higher mating frequencies and shorter latencies than did group-housed flies (Bastock and Manning, 1955; Ellis and Kessler, 1975). Maynard Smith (1956) showed that D. subobscura males reared in isolation were more successful in mating than those reared in groups. Isolation of D. silvestris decreased mating success significantly (Sene, 1977), however. Here, effects of preadult seclusion on the D. paulistorum mate choices varied with semispecies, with stage, and with gender of the treated flies as summarized below.

| Test pair | Semispecies & sex secluded | Effect of Seclusion | I |
|---|---|---|---|
| CA♀ x OR♂ (0.68±0.07) | CA♀♀ | no effect | 0 |
| | CA♂♂ | S1 - S3 | - |
| | CA♀♀,♂♂ | S1 - S2 | - |
| OR♀ x CA♂ (0.40±0.09) | OR♀♀ | no effect | 0 |
| | OR♂♂ | no effect | 0 |
| | OR♀♀,♂♂ | no effect | 0 |
| AN♀ x TR♂ (0.96±0.03) | AN♀♀ | S1 | - |
| | AN♂♂ | S1 - S2 | - |
| | AN♀♀,♂♂ | S1 - S2 | - |
| TR♀ x AN♂ (0.54±0.08) | TR♀♀ | no effect | 0 |
| | TR♂♂ | no effect | 0 |
| | TR♀♀,♂♂ | no effect | 0 |
| AM♀ x IN♂ (0.96±0.03) | AM♀♀ | S1 - S2 | - |
| | AM♂♂ | S1 - S2 | - |
| | AM♀♀,♂♂ | S1 | - |
| IN♀ x AM♂ (0.98±0.02) | IN♀♀ | S1 | - |
| | IN♂♂ | no effect | 0 |
| | IN♀♀,♂♂ | no effect | 0 |

-, isolation was significantly decreased; 0, isolation was not significantly changed.

Sexual isolation between CA and OR was incomplete and asymmetric ($I_{CA♀,OR♂} = 0.68$; $I_{OR♀,CA♂} = 0.40$; $\underline{X}^2 = 4.77$, $p \leq 0.05$). Female CA were not influenced by seclusion, but preadult seclusion influenced mating patterns of male CA tested with OR and this seclusion effect was strong enough to alter the mate choice of CA when both sexes were secluded. However, the reciprocal cross, OR♀♀ x (OR♂♂ + CA♂♂), showed a low degree of sexual isolation, and mating patterns of OR were not influenced by any of three kinds of seclusion. The degree of isolation of types, AN♀♀ x (AN♂♂ + TR♂♂), is almost perfect ($I_{AN♀,TR♂}$ = 0.96). Mating patterns of AN were significantly influenced by seclusion. While the degree of isolation of the reciprocal cross was low ($I_{TR♀,AN♂}$ = 0.54), seclusion did not modify the mating behaviors of TR semispecies. In another set, AM and IN, which also showed a high degree of sexual isolation, mating patterns of AM were influenced by three types of seclusion, while only female preadult seclusion influenced mate choices of IN. Therefore, seclusion affected D. paulistorum mating behavior only when the two semispecies involved were highly reproductively isolated. The seclusion effect was stronger at early developmental stages than at later stages. Discriminatory abilities were reduced, and therefore sexual isolation indices significantly decreased, when individuals were totally secluded from their consemispecifics at early stages of development. There was no difference between sexes in vulnerability to seclusion, except for the CA semispecies where males were more vulnerable to seclusion than females ($F_{1,10}$ = 6.76, $p \leq 0.05$). It is interesting to note that mating patterns of semispecies, after seclusion of both sexes from the same semispecies, generally follows the secluded-male-only behaviors. Seclusion also significantly affected intrasemispecific choices and homogamic matings were more frequent than heterogamic matings (Table V); flies were more likely to mate with flies having experiences similar to their own, i.e, raised together or secluded.

Heterosemispecific contacts, LTCVC, PIC and mixed culturing, resulted in different degrees of alteration of mating behaviors. When the semispecies were subjected to long term chemical and vibratory contact, their intersemispecific choices were not significantly changed during the initial generations, but ultimately isolation indices significantly increased. The number of generations taken to attain a perfect isolation index varied with the pair of semispecies tested: Ten generations for CA and OR; four generations for AM and IN. Isolation indices between AN and TR remained around 0.70, however, even after 10 generations (Table VI).

Short exposure to another semispecies via PIC did not always influence mating choices, but, again when AN and TR were tested, isolation indices significantly decreased in three combinations (Table VII). The failure of PIC to alter mating behaviors was possibly indicative of a short period of exposure to heterosemispecifics after emergence (Williams and Lenington, 1993). Some studies show that sexual preference acqruied from conspecifics during a sensitive period can be altered by later experiences (Immelman et al., 1991; Kruijt and Meeuwissen, 1991). The lack of altered behavior could also be attributed to the nearly perfect initial isolation between the two semispecies. It is interesting to note here that TR was certainly behaved as a transitional entity in my experiments: 1) TR was partially isolated from AN ($I_{TR♀,AN♂}$ = 0.54); and 2) TR was also vulnerable to both LTCVC and PIC; in pairing with AN, the numbers of heterogamic matings significantly increased.

Mixed culturing had its greatest effects on sexual isolation when control populations were not strongly isolated. Then, discriminatory abilities increased in the artificially sympatric setting. Mutant AN vs. CA (Table VIII) is one example of this: Isolation significantly increased at the generation following initiation of mixed culturing (Set B, Table VIII; Fig. VIII); and isolation indices were highly significant compared to those of controls across twenty-two generations. When selection was reinstated five generations after relaxation (Set D & E, Table VIII), discriminatory abilities resumed and isolation indices again increased. Furthermore, there was very strong sexual isolation between Control (parent) and Selected (daughter) lines subjected to mixed culturing (Set F & G, Table VIII). Therefore, <u>different forms of artificial selection consistently resulted in the reinforcement of sexual isolation</u>, which justifies the Muller-Mayr view about the origin of sexual isolation.

Several studies have reported that mixed culturing influences sexual isolation between strains of the same or of different species. Wallace (1954), Knight et al. (1956), Ehrman (1971, 1983) and Crossley (1974) have demonstrated that mating preferences of <u>Drosophila</u> populations can be modified by selection. Mayr and Dobzhansky (1945) and Koopman (1950) found increased isolation between <u>D</u>. <u>pseudoobscura</u> and <u>D</u>.

*persimilis* after rearing them in mixed cultures. Kessler (1966) extended Koopman's results by showing that artificial selection significantly increased or decreased the isolation between these same two sibling species, depending upon the direction of selection. It has been shown that sexual isolation between New Llanos and Orinocan strains of *D. paulistorum* could be constructed where none had existed before initiation of selection for it (Dobzhansky and Pavlovsky, 1971; Dobzhansky et al. 1976). In addition, several studies of natural populations have yielded strong evidence for the reinforcement of sexual isolation via selection against hybrids: *D. mojavensis* (Wasserman and Koepfer, 1977, 1980; Zouros and d'Entremont, 1980; Markow, 1991; Markow et al., 1983); *D. paulistorum* (Ehrman, 1965); damselflies (Waage, 1975, 1979); frogs (Fouquette, 1975; Littlejohn and Loftus-Hills, 1968); ground crickets (Howard, 1986; Benedix and Howard, 1991); mosquitoes (McLain and Rai, 1986); and salamanders (Uzendoski and Verrell, 1993). Also see Butlin (1989) for a critical review. Wasserman and Koepfer (1977) demonstrated that *D. mojavensis* exhibited a high level of sexual isolation from sympatric *D. arizonensis* in Sonora, and that sexual isolation between Baja Californian and Sonoran populations in *D. mojavensis* was viewed as the outcome of sympatric interactions with *D. arizonensis*. *D. paulistorum* semispecies overlap geographically in nature. Therefore, larvae of two or more *D. paulistorum* semispecies may develop together. Such sympatry results in enhancement of mate discrimination and an increase in isolation between sympatric semispecies; this is clearly of survival value to *D. paulistorum*.

## CONCLUSIONS

Preadult seclusion exerted significant effects on mate choices and, as a consequence, decreased sexual isolation significantly, while artificial selection including LTCVC and mixed culturing reinforced sexual isolation. Long term chemical and vibratory contact was strong enough to increase sexual isolation between semispecies, though it took longer intervals compared to a direct effect of mixed culturing. Postimaginal contact was relatively ineffective in altering mating behavior. Effective population size of D. paulistorum would undoubtedly be influenced by these preadult experiences in nature. Efforts to create "tissue markers" with beta-gal (Bellen et al., 1989) are now proceeding, in order to distinguish these six morphologically-identical sibling semispecies without the use of often debilitating mutant markers. In this way I hope to eliminate problems created by such mutant markers with their pleiotropic effects on mating behavior and on viability, in general.

Table I. Seclusion experiments in *Drosophila paulistorum*: Rearing methods for control (CT) and experimental (S1 - S6) groups.

| Stage | egg | 1st instar | 2nd instar | 3rd instar | pupa | adult |
|---|---|---|---|---|---|---|
| Group | | | | | | |
| CT | C | C | C | C | C | C |
| S6 | C | C | C | C | C | S |
| S5 | C | C | C | C | S | S |
| S4 | C | C | C | S | S | S |
| S3 | C | C | S | S | S | S |
| S2 | C | S | S | S | S | S |
| S1 | S | S | S | S | S | S |

C = Communal: in mass culture during the indicated stage(s)
S = Secluded: physically isolated during the indicated stage(s)

Table II. Effect of seclusion on mate choices in *Drosophila paulistorum* semispecies from Honduras (CA) and Guyana (OR): Results of female choice tests.

| Subset | Female | Ho Male | He Male | Matings Ho | Matings He | I±SE | S vs C |
|---|---|---|---|---|---|---|---|
| A1) | CA C | CA C | OR C | 84 | 16 | 0.68±0.07 | |
| | CA S6 | CA C | OR C | 81 | 19 | 0.62±0.08 | ns |
| | CA S5 | CA C | OR C | 74 | 26 | 0.48±0.09 | ns |
| | CA S4 | CA C | OR C | 72 | 28 | 0.44±0.09 | ns |
| | CA S3 | CA C | OR C | 71 | 29 | 0.42±0.09 | ns |
| | CA S2 | CA C | OR C | 68 | 32 | 0.36±0.09 | ns |
| | CA S1 | CA C | OR C | 65 | 35 | 0.30±0.10 | ns |
| A2) | CA C | CA C | OR C | 84 | 16 | 0.68±0.07 | |
| | CA C | CA S6 | OR C | 79 | 21 | 0.58±0.08 | ns |
| | CA C | CA S5 | OR C | 71 | 29 | 0.42±0.09 | ns |
| | CA C | CA S4 | OR C | 68 | 32 | 0.36±0.09 | ns |
| | CA C | CA S3 | OR C | 51 | 49 | 0.02±0.10 | * |
| | CA C | CA S2 | OR C | 50 | 50 | 0.00±0.10 | * |
| | CA C | CA S1 | OR C | 49 | 51 | -0.02±0.10 | ** |
| A3) | CA C | CA C | OR C | 84 | 16 | 0.68±0.07 | |
| | CA S6 | CA S6 | OR C | 80 | 20 | 0.60±0.08 | ns |
| | CA S5 | CA S5 | OR C | 73 | 27 | 0.46±0.09 | ns |
| | CA S4 | CA S4 | OR C | 69 | 31 | 0.38±0.09 | ns |
| | CA S3 | CA S3 | OR C | 64 | 36 | 0.28±0.10 | ns |
| | CA S2 | CA S2 | OR C | 51 | 49 | 0.02±0.10 | * |
| | CA S1 | CA S1 | OR C | 49 | 51 | -0.02±0.10 | ** |

Table II (continued)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| B1) | OR C | OR C | CA C | 70 | 30 | 0.40±0.09 | |
| | OR S6 | OR C | CA C | 69 | 31 | 0.38±0.09 | ns |
| | OR S5 | OR C | CA C | 68 | 32 | 0.36±0.09 | ns |
| | OR S4 | OR C | CA C | 66 | 34 | 0.32±0.10 | ns |
| | OR S3 | OR C | CA C | 63 | 37 | 0.26±0.10 | ns |
| | OR S2 | OR C | CA C | 62 | 38 | 0.24±0.10 | ns |
| | OR S1 | OR C | CA C | 61 | 39 | 0.22±0.10 | ns |
| B2) | OR C | OR C | CA C | 70 | 30 | 0.40±0.09 | |
| | OR C | OR S6 | CA C | 54 | 46 | 0.08±0.10 | ns |
| | OR C | OR S5 | CA C | 52 | 48 | 0.04±0.10 | * |
| | OR C | OR S4 | CA C | 55 | 45 | 0.10±0.10 | ns |
| | OR C | OR S3 | CA C | 60 | 40 | 0.20±0.10 | ns |
| | OR C | OR S2 | CA C | 60 | 40 | 0.20±0.10 | ns |
| | OR C | OR S1 | CA C | 56 | 44 | 0.12±0.10 | ns |
| B3) | OR C | OR C | CA C | 70 | 30 | 0.40±0.09 | |
| | OR S6 | OR S6 | CA C | 69 | 31 | 0.38±0.09 | ns |
| | OR S5 | OR S5 | CA C | 67 | 33 | 0.34±0.09 | ns |
| | OR S4 | OR S4 | CA C | 65 | 35 | 0.30±0.10 | ns |
| | OR S3 | OR S3 | CA C | 64 | 36 | 0.28±0.10 | ns |
| | OR S2 | OR S2 | CA C | 62 | 38 | 0.24±0.10 | ns |
| | OR S1 | OR S1 | CA C | 60 | 40 | 0.20±0.10 | ns |

C, communally-raised controls; S, secluded; I, isolation index; Ho, homogamic; He, heterogamic; significance of the difference between a given S combination and its corresponding control combination is based on the T-method. The minimum significant ranges between any pair of mean isolation indices within subgroups are as follows: A1) 0.65 (5%); A2) 0.59 (5%), 0.72 (1%); A3) 0.57 (5%), 0.70 (1%); B1) 0.40 (5%); B2) 0.36 (5%); B3) 0.32 (5%); * $p \leq 0.05$, ** $p \leq 0.01$.

Table III. Effect of seclusion on mate choices in <u>Drosophila paulistorum</u> semispecies from Mesitas, Colombia (AN) and Santa Marta, Colombia (TR): Results of female choice tests.

| Subset | Female | Ho Male | He Male | Matings Ho | He | I±SE | S vs C |
|---|---|---|---|---|---|---|---|
| A1) | AN C | AN C | TR C | 98 | 2 | 0.96±0.03 | |
| | AN S6 | AN C | TR C | 97 | 3 | 0.94±0.03 | ns |
| | AN S5 | AN C | TR C | 91 | 9 | 0.82±0.06 | ns |
| | AN S4 | AN C | TR C | 90 | 10 | 0.80±0.06 | ns |
| | AN S3 | AN C | TR C | 84 | 16 | 0.68±0.07 | ns |
| | AN S2 | AN C | TR C | 75 | 25 | 0.50±0.09 | ns |
| | AN S1 | AN C | TR C | 67 | 23 | 0.34±0.09 | * |
| A2) | AN C | AN C | TR C | 98 | 2 | 0.96±0.03 | |
| | AN C | AN S6 | TR C | 96 | 4 | 0.92±0.04 | ns |
| | AN C | AN S5 | TR C | 91 | 9 | 0.82±0.06 | ns |
| | AN C | AN S4 | TR C | 91 | 9 | 0.82±0.06 | ns |
| | AN C | AN S3 | TR C | 83 | 17 | 0.66±0.08 | ns |
| | AN C | AN S2 | TR C | 70 | 30 | 0.40±0.09 | ** |
| | AN C | AN S1 | TR C | 70 | 30 | 0.40±0.09 | ** |
| A3) | AN C | AN C | TR C | 98 | 2 | 0.96±0.03 | |
| | AN S6 | AN S6 | TR C | 95 | 5 | 0.90±0.04 | ns |
| | AN S5 | AN S5 | TR C | 94 | 6 | 0.88±0.05 | ns |
| | AN S4 | AN S4 | TR C | 90 | 10 | 0.80±0.06 | ns |
| | AN S3 | AN S3 | TR C | 80 | 20 | 0.60±0.08 | ns |
| | AN S2 | AN S2 | TR C | 69 | 31 | 0.38±0.09 | * |
| | AN S1 | AN S1 | TR C | 72 | 28 | 0.44±0.09 | * |

Table III (continued)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| B1) | TR C | TR C | AN C | 77 | 23 | 0.54±0.08 | |
| | TR S6 | TR C | AN C | 74 | 26 | 0.48±0.09 | ns |
| | TR S5 | TR C | AN C | 75 | 25 | 0.50±0.09 | ns |
| | TR S4 | TR C | AN C | 75 | 25 | 0.50±0.09 | ns |
| | TR S3 | TR C | AN C | 74 | 26 | 0.48±0.09 | ns |
| | TR S2 | TR C | AN C | 75 | 25 | 0.50±0.09 | ns |
| | TR S1 | TR C | AN C | 70 | 30 | 0.40±0.09 | ns |
| B2) | TR C | TR C | AN C | 77 | 23 | 0.54±0.08 | |
| | TR C | TR S6 | AN C | 74 | 26 | 0.48±0.09 | ns |
| | TR C | TR S5 | AN C | 73 | 27 | 0.46±0.09 | ns |
| | TR C | TR S4 | AN C | 69 | 31 | 0.38±0.09 | ns |
| | TR C | TR S3 | AN C | 71 | 29 | 0.42±0.09 | ns |
| | TR C | TR S2 | AN C | 62 | 38 | 0.24±0.10 | ns |
| | TR C | TR S1 | AN C | 64 | 36 | 0.28±0.10 | ns |
| B3) | TR C | TR C | AN C | 77 | 23 | 0.54±0.08 | |
| | TR S6 | TR S6 | AN C | 74 | 26 | 0.48±0.09 | ns |
| | TR S5 | TR S5 | AN C | 72 | 28 | 0.44±0.09 | ns |
| | TR S4 | TR S4 | AN C | 71 | 29 | 0.42±0.09 | ns |
| | TR S3 | TR S3 | AN C | 72 | 28 | 0.44±0.09 | ns |
| | TR S2 | TR S2 | AN C | 64 | 36 | 0.28±0.10 | ns |
| | TR S1 | TR S1 | AN C | 66 | 34 | 0.32±0.10 | ns |

C, communally-raised controls; S, secluded; I, isolation index; Ho, homogamic; He, heterogamic; significance of the difference between a given S combination and its corresponding control combination is based on the T-method. The minimum significant ranges between any pair of mean isolation indices within subgroups are as follows: A1) 0.54 (5%); A2) 0.45 (5%), 0.56 (1%); A3) 0.52 (5%); B1) 0.70 (5%); B2) 0.77 (5%); B3) 0.68 (5%); * $p \leq 0.05$, ** $p \leq 0.01$.

Table IV. Effect of seclusion on mating choice in _Drosophila_ _paulistorum_ semispecies from Brazil (AM) and Colombia (IN): Results of female choice tests.

| Subset | Female | Ho Male | He Male | Matings Ho | He | I±SE | S vs C |
|---|---|---|---|---|---|---|---|
| A1) | AM C | AM C | IN C | 98 | 2 | 0.96±0.03 | |
| | AM S6 | AM C | IN C | 90 | 10 | 0.80±0.06 | ns |
| | AM S5 | AM C | IN C | 86 | 14 | 0.72±0.07 | ns |
| | AM S4 | AM C | IN C | 85 | 15 | 0.70±0.07 | ns |
| | AM S3 | AM C | IN C | 84 | 16 | 0.68±0.07 | ns |
| | AM S2 | AM C | IN C | 74 | 26 | 0.48±0.09 | ** |
| | AM S1 | AM C | IN C | 70 | 30 | 0.40±0.09 | ** |
| A2) | AM C | AM C | IN C | 98 | 2 | 0.96±0.03 | |
| | AM C | AM S6 | IN C | 95 | 5 | 0.90±0.04 | ns |
| | AM C | AM S5 | IN C | 89 | 11 | 0.78±0.06 | ns |
| | AM C | AM S4 | IN C | 88 | 12 | 0.76±0.07 | ns |
| | AM C | AM S3 | IN C | 86 | 14 | 0.72±0.07 | ns |
| | AM C | AM S2 | IN C | 79 | 21 | 0.58±0.08 | ** |
| | AM C | AM S1 | IN C | 79 | 21 | 0.58±0.08 | ** |
| A3) | AM C | AM C | IN C | 98 | 2 | 0.96±0.03 | |
| | AM S6 | AM S6 | IN C | 97 | 3 | 0.94±0.03 | ns |
| | AM S5 | AM S5 | IN C | 93 | 7 | 0.86±0.05 | ns |
| | AM S4 | AM S4 | IN C | 89 | 11 | 0.78±0.06 | ns |
| | AM S3 | AM S3 | IN C | 88 | 12 | 0.76±0.07 | ns |
| | AM S2 | AM S2 | IN C | 88 | 12 | 0.76±0.07 | ns |
| | AM S1 | AM S1 | IN C | 82 | 18 | 0.64±0.08 | * |

Table IV (continued)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| B1) | IN C | IN C | AM C | 99 | 1 | 0.98±0.02 | |
| | IN S6 | IN C | AM C | 97 | 3 | 0.94±0.03 | ns |
| | IN S5 | IN C | AM C | 97 | 3 | 0.94±0.03 | ns |
| | IN S4 | IN C | AM C | 98 | 2 | 0.96±0.03 | ns |
| | IN S3 | IN C | AM C | 92 | 8 | 0.84±0.05 | ns |
| | IN S2 | IN C | AM C | 80 | 20 | 0.60±0.08 | ns |
| | IN S1 | IN C | AM C | 79 | 21 | 0.58±0.08 | * |
| B2) | IN C | IN C | AM C | 99 | 1 | 0.98±0.02 | |
| | IN C | IN S6 | AM C | 99 | 1 | 0.98±0.02 | ns |
| | IN C | IN S5 | AM C | 98 | 2 | 0.96±0.03 | ns |
| | IN C | IN S4 | AM C | 95 | 5 | 0.90±0.04 | ns |
| | IN C | IN S3 | AM C | 96 | 4 | 0.92±0.04 | ns |
| | IN C | IN S2 | AM C | 96 | 4 | 0.92±0.04 | ns |
| | IN C | IN S1 | AM C | 94 | 6 | 0.88±0.05 | ns |
| B3) | IN C | IN C | AM C | 99 | 1 | 0.98±0.02 | |
| | IN S6 | IN S6 | AM C | 98 | 2 | 0.96±0.03 | ns |
| | IN S5 | IN S5 | AM C | 95 | 5 | 0.90±0.04 | ns |
| | IN S4 | IN S4 | AM C | 95 | 5 | 0.90±0.04 | ns |
| | IN S3 | IN S3 | AM C | 96 | 4 | 0.92±0.04 | ns |
| | IN S2 | IN S2 | AM C | 96 | 4 | 0.92±0.04 | ns |
| | IN S1 | IN S1 | AM C | 94 | 6 | 0.88±0.05 | ns |

C, communally-raised controls; S, secluded; I, isolation index; Ho, homogamic; He, heterogamic; significance of the difference between a given S combination and its corresponding control combination is based on the T-method. The minimum significant ranges between any pair of mean isolation indices within subgroups are as follows: A1) 0.29 (5%), 0.35 (1%); A2) 0.27 (5%), 0.33 (1%); A3) 0.28 (5%); B1) 0.39 (5%); B2) 0.20 (5%); B3) 0.16 (5%); * $p \leq 0.05$, ** $p \leq 0.01$.

Table V. Effect of seclusion on intrasemispecific mate choices in <u>Drosophila paulistorum</u> semispecies: Results of multiple choice tests.

| Test Set | Stage of S Individuals | Mating Combinations | | | | I±SE | Ic±SE | Is±SE |
|---|---|---|---|---|---|---|---|---|
| | | C♀xC♂ | C♀xS♂ | S♀xC♂ | S♀xS♂ | | | |
| A) CA C x CA S | S6 | 36 | 24 | 32 | 28 | 0.07±0.09 | 0.20±0.13 | 0.07±0.13 |
| | S5 | 32 | 28 | 15 | 45 | 0.28±0.09** | 0.07±0.13 | 0.56±0.11** |
| | S4 | 43 | 17 | 17 | 43 | 0.43±0.08** | 0.43±0.12** | 0.43±0.12** |
| | S3 | 47 | 13 | 30 | 30 | 0.28±0.09** | 0.57±0.11** | 0.00±0.13 |
| | S2 | 48 | 12 | 29 | 31 | 0.32±0.09** | 0.60±0.10** | 0.03±0.13 |
| | S1 | 33 | 27 | 18 | 42 | 0.25±0.09* | 0.10±0.13 | 0.40±0.12** |
| B) OR C x OR S | S6 | 34 | 26 | 29 | 31 | 0.08±0.09 | 0.13±0.13 | 0.03±0.13 |
| | S5 | 28 | 32 | 30 | 30 | -0.03±0.09 | -0.07±0.13 | 0.00±0.13 |
| | S4 | 33 | 27 | 28 | 32 | 0.08±0.09 | 0.10±0.13 | 0.07±0.13 |
| | S3 | 43 | 17 | 23 | 37 | 0.33±0.09** | 0.43±0.12** | 0.23±0.13 |
| | S2 | 32 | 28 | 17 | 43 | 0.25±0.09* | 0.07±0.13 | 0.43±0.12** |
| | S1 | 33 | 27 | 29 | 31 | 0.07±0.09 | 0.10±0.13 | 0.03±0.13 |
| C) AN C x AN S | S6 | 42 | 18 | 21 | 39 | 0.35±0.09** | 0.40±0.12** | 0.30±0.12* |
| | S5 | 37 | 23 | 21 | 39 | 0.27±0.09* | 0.23±0.13 | 0.30±0.12* |
| | S4 | 37 | 23 | 25 | 35 | 0.20±0.09* | 0.23±0.13 | 0.17±0.13 |
| | S3 | 40 | 20 | 27 | 33 | 0.22±0.09* | 0.33±0.12* | 0.10±0.13 |
| | S2 | 42 | 18 | 29 | 31 | 0.22±0.09* | 0.40±0.12** | 0.03±0.13 |
| | S1 | 45 | 15 | 35 | 25 | 0.17±0.09 | 0.50±0.11** | -0.17±0.13 |

Table V (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| D) TR C x TR S | S6 | 41 | 19 | 23 | 37 | 0.30±0.09** | 0.37±0.12** | 0.23±0.13 |
| | S5 | 23 | 37 | 19 | 41 | 0.07±0.09 | -0.23±0.13 | 0.37±0.12* |
| | S4 | 35 | 25 | 27 | 33 | 0.13±0.09 | 0.17±0.13 | 0.10±0.13 |
| | S3 | 41 | 19 | 28 | 32 | 0.22±0.09* | 0.37±0.12** | 0.07±0.13 |
| | S2 | 46 | 14 | 37 | 23 | 0.15±0.09 | 0.53±0.11** | -0.23±0.13 |
| | S1 | 46 | 14 | 49 | 11 | -0.05±0.09 | 0.53±0.11** | -0.63±0.10** |
| E) AM C x AM S | S6 | 39 | 21 | 21 | 39 | 0.30±0.09** | 0.30±0.12* | 0.30±0.12* |
| | S5 | 47 | 13 | 27 | 33 | 0.33±0.09** | 0.57±0.11** | 0.10±0.13 |
| | S4 | 32 | 28 | 19 | 41 | 0.22±0.09* | 0.07±0.13 | 0.37±0.12** |
| | S3 | 41 | 19 | 20 | 40 | 0.35±0.09** | 0.37±0.12** | 0.33±0.12* |
| | S2 | 32 | 28 | 22 | 38 | 0.17±0.09 | 0.07±0.13 | 0.27±0.12* |
| | S1 | 45 | 15 | 30 | 30 | 0.25±0.09* | 0.50±0.11** | 0.00±0.13 |
| F) IN C x IN S | S6 | 31 | 29 | 24 | 36 | 0.12±0.09 | 0.03±0.13 | 0.20±0.13 |
| | S5 | 41 | 19 | 29 | 31 | 0.20±0.09* | 0.37±0.12** | 0.03±0.13 |
| | S4 | 28 | 32 | 29 | 31 | -0.02±0.09 | 0.07±0.13 | 0.03±0.13 |
| | S3 | 31 | 29 | 33 | 27 | -0.03±0.09 | 0.03±0.13 | 0.10±0.13 |
| | S2 | 41 | 19 | 25 | 35 | 0.27±0.09* | 0.37±0.12** | 0.17±0.13 |
| | S1 | 40 | 20 | 22 | 38 | 0.30±0.09** | 0.33±0.12* | 0.27±0.12* |

C, communally-raised controls; S, secluded; I, isolation index; Ic, female isolation index of controls; Is, female isolation index of secludes; there is no significant heterogeneity within each of subsets; * $p<0.05$, ** $p<0.01$.

Table VI. Long term chemical and vibratory contact experiments in *Drosophila paulistorum* semispecies: Results of multiple choice tests.

| Test Set | No. of generations of LTCVC | Multiple Choice Mating Combinations | | | | I±SE | LTCVC vs C |
|---|---|---|---|---|---|---|---|
| A) CA x OR | | CA♀xCA♂ | CA♀xOR♂ | OR♀xCA♂ | OR♀xOR♂ | | |
| | 1 | 56 | 4 | 8 | 52 | 0.80±0.05 | ns |
| | 2 | 56 | 4 | 3 | 57 | 0.88±0.04 | ns |
| | 4 | 54 | 6 | 7 | 53 | 0.78±0.06 | ns |
| | 6 | 54 | 6 | 6 | 54 | 0.80±0.05 | ns |
| | 8 | 56 | 4 | 9 | 51 | 0.78±0.06 | ns |
| | 10 | 60 | 0 | 0 | 60 | 1.00±0.00 | * |
| | Control | 50 | 10 | 9 | 51 | 0.68±0.07 | |
| B) AN x TR | | AN♀xAN♂ | AN♀xTR♂ | TR♀xAN♂ | TR♀xTR♂ | | |
| | 1 | 54 | 6 | 9 | 51 | 0.75±0.06 | ns |
| | 2 | 58 | 2 | 2 | 58 | 0.93±0.03 | ns |
| | 4 | 55 | 5 | 11 | 49 | 0.73±0.06 | ns |
| | 6 | 50 | 10 | 2 | 58 | 0.80±0.05 | ns |
| | 8 | 57 | 3 | 10 | 50 | 0.78±0.06 | ns |
| | 10 | 55 | 5 | 14 | 46 | 0.68±0.07 | ns |
| | Control | 59 | 1 | 1 | 59 | 0.97±0.02 | |

Table VI (continued)

| C) AM x IN | | AM♀xAM♂ | AM♀xIN♂ | IN♀xAM♂ | IN♀xIN♂ | | |
|---|---|---|---|---|---|---|---|
| | 1 | 59 | 1 | 5 | 55 | 0.90±0.04 | ns |
| | 2 | 60 | 0 | 4 | 56 | 0.93±0.03 | ns |
| | 4 | 60 | 0 | 0 | 60 | 1.00±0.00 | ns |
| | 6 | 60 | 0 | 0 | 60 | 1.00±0.00 | ns |
| | 8 | 60 | 0 | 0 | 60 | 1.00±0.00 | ns |
| | 10 | 60 | 0 | 0 | 60 | 1.00±0.00 | ns |
| | Control | 59 | 1 | 1 | 59 | 0.97±0.02 | |

C, communally-raised; LTCVC, one semispecies was connected with another semispecies through a fritted glass bridge during a whole life cycle; significance of the difference between a given generation of LTCVC and the control is based on the T-method. The minimum significant ranges between any pair of mean isolation indices within subgroups are as follows: A) 0.30 (5%); B) 0.33 (5%); C) 0.09 (5%); * p≤0.05.

Table VII. Postimaginal contact experiments in *Drosophila paulistorum* semispecies: Results of multiple choice tests.

| Test Set | Semispecies & Sex with PIC | Multiple Choice Mating Combinations | | | | I±SE | PIC vs C |
|---|---|---|---|---|---|---|---|
| A) CA x OR | | CA♀xCA♂ | CA♀xOR♂ | OR♀xCA♂ | OR♀xOR♂ | | |
| | CA♀ | 50 | 10 | 14 | 46 | 0.60±0.07 | ns |
| | CA♂ | 54 | 6 | 6 | 54 | 0.80±0.05 | ns |
| | CA♀,CA♂ | 49 | 11 | 18 | 42 | 0.52±0.08 | ns |
| | OR♀ | 50 | 10 | 18 | 42 | 0.53±0.08 | ns |
| | OR♂ | 47 | 13 | 21 | 39 | 0.43±0.08 | ns |
| | OR♀,OR♂ | 43 | 17 | 10 | 50 | 0.55±0.08 | ns |
| | CA♀,CA♂,OR♀,OR♂ | 43 | 17 | 15 | 45 | 0.47±0.08 | ns |
| | Control | 50 | 10 | 9 | 51 | 0.68±0.07 | |
| B) AN x TR | | AN♀xAN♂ | AN♀xTR♂ | TR♀xAN♂ | TR♀xTR♂ | | |
| | AN♀ | 56 | 4 | 2 | 58 | 0.90±0.04 | ns |
| | AN♂ | 59 | 1 | 5 | 55 | 0.90±0.04 | ns |
| | AN♀,AN♂ | 59 | 1 | 1 | 59 | 0.97±0.02 | ns |
| | TR♀ | 44 | 16 | 17 | 43 | 0.45±0.08 | ** |
| | TR♂ | 48 | 12 | 15 | 45 | 0.55±0.08 | ** |
| | TR♀,TR♂ | 43 | 17 | 12 | 48 | 0.52±0.08 | ** |
| | AN♀,AN♂,TR♀,TR♂ | 56 | 4 | 3 | 57 | 0.88±0.04 | ns |
| | Control | 59 | 1 | 1 | 59 | 0.97±0.02 | |

Table VII (continued)

| C) AM x IN | AM♀xAM♂ | AM♀xIN♂ | IN♀xAM♂ | IN♀xIN♂ | | |
|---|---|---|---|---|---|---|
| AM♀ | 60 | 0 | 2 | 58 | 0.96±0.03 | ns |
| AM♂ | 60 | 0 | 1 | 59 | 0.98±0.02 | ns |
| AM♀,AM♂ | 59 | 1 | 9 | 51 | 0.83±0.05 | ns |
| IN♀ | 60 | 0 | 10 | 50 | 0.83±0.05 | ns |
| IN♂ | 60 | 0 | 2 | 58 | 0.97±0.02 | ns |
| IN♀,IN♂ | 60 | 0 | 4 | 56 | 0.93±0.03 | ns |
| AM♀,AM♂,IN♀,IN♂ | 58 | 2 | 6 | 54 | 0.87±0.04 | ns |
| Control | 59 | 1 | 1 | 59 | 0.97±0.02 | |

C, communally-raised; PIC, the indicated semispecies and gender were placed with adults of another semispecies but with the same sex for three days; significance of the difference between a given PIC combination and the control is based on the T-method. The minimum significant ranges between any pair of mean isolation indices within subgroups are as follows: A) 0.35 (5%); B) 0.30 (5%); C) 0.22 (5%); ** $p \leq 0.01$.

Table VIII. The influence of method of rearing on sexual isolation between two <u>Drosophila</u> <u>paulistorum</u> semispecies, Honduras (CA) and Colombia (AN): Results of multiple choice tests.

| | Multiple Choice Mating Combinations | | | | | |
|---|---|---|---|---|---|---|
| | CA♀xCA♂ | CA♀xAN♂ | AN♀xCA♂ | AN♀xAN♂ | $X^2_{♂}$ | I±SE |
| A1) CONTROLS (Mutants) | | | | | | |
| at Gen 0 | 34 | 26 | 0 | 60 | 22.53** | 0.57±0.08 |
| at Gen 20 | 33 | 27 | 0 | 60 | 24.30** | 0.55±0.08 |
| at Gen 43 | 34 | 26 | 0 | 60 | 22.53** | 0.57±0.08 |
| A2) CONTROLS (Wildtypes) | | | | | | |
| at Gen 0 | 54 | 6 | 7 | 53 | 0.03 | 0.78±0.06 |
| B) SELECTED (Mixed Culturing) | | | | | | |
| at Gen 1 | 55 | 5 | 1 | 59 | 0.53 | 0.90±0.04 |
| at Gen 2 | 54 | 6 | 0 | 60 | 1.20 | 0.90±0.04 |
| at Gen 4 | 54 | 6 | 0 | 60 | 1.20 | 0.90±0.04 |
| at Gen 6 | 60 | 0 | 1 | 59 | 0.00 | 0.98±0.02 |
| at Gen 8 | 60 | 0 | 3 | 57 | 0.30 | 0.95±0.03 |
| at Gen 10 | 60 | 0 | 5 | 55 | 0.83 | 0.92±0.04 |
| at Gen 12 | 53 | 7 | 3 | 57 | 0.53 | 0.83±0.05 |
| at Gen 14 | 58 | 2 | 0 | 60 | 0.13 | 0.97±0.02 |
| at Gen 15 | 55 | 5 | 0 | 60 | 0.83 | 0.92±0.04 |
| at Gen 17 | 58 | 2 | 1 | 59 | 0.03 | 0.95±0.03 |
| at Gen 19 | 55 | 5 | 0 | 60 | 0.83 | 0.92±0.04 |
| at Gen 20 | 55 | 5 | 1 | 59 | 0.53 | 0.90±0.04 |
| at Gen 22 | 54 | 6 | 0 | 60 | 1.20 | 0.90±0.04 |
| at Gen 43 | 56 | 4 | 1 | 59 | 0.30 | 0.92±0.04 |

Table VIII (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| C) | RELAXED AT GEN 14 | | | | | |
| | at Gen 15 | 52 | 8 | 0 | 60 | 2.13 | 0.87±0.05 |
| | at Gen 16 | 46 | 14 | 10 | 50 | 0.53 | 0.60±0.07 |
| | at Gen 17 | 29 | 31 | 0 | 60 | 32.03** | 0.48±0.08 |
| | at Gen 18 | 42 | 18 | 14 | 46 | 0.53 | 0.47±0.08 |
| | at Gen 19 | 36 | 24 | 4 | 56 | 13.33** | 0.53±0.08 |
| | at Gen 20 | 44 | 16 | 13 | 47 | 0.30 | 0.52±0.08 |
| | at Gen 22 | 39 | 21 | 8 | 52 | 5.63* | 0.52±0.08 |
| D) | RESELECTED AT GEN 19 (RS-1) | | | | | | |
| | at Gen 20 | 53 | 7 | 6 | 54 | 0.03 | 0.78±0.06 |
| | at Gen 21 | 51 | 9 | 2 | 58 | 1.63 | 0.82±0.05 |
| | at Gen 22 | 54 | 6 | 1 | 59 | 0.83 | 0.88±0.04 |
| E) | RESELECTED AT GEN 19 (RS-2) | | | | | | |
| | at Gen 20 | 49 | 11 | 5 | 55 | 1.20 | 0.73±0.06 |
| | at Gen 21 | 51 | 9 | 5 | 55 | 0.53 | 0.77±0.06 |
| | at Gen 22 | 55 | 5 | 1 | 59 | 0.53 | 0.90±0.04 |
| F) | BETWEEN CA (C) AND CA (S) | | | | | | |
| | | C♀xC♂ | C♀xS♂ | S♀xC♂ | S♀xS♂ | | |
| | at Gen 43 | 58 | 2 | 17 | 43 | 7.50** | 0.68±0.07 |
| G) | BETWEEN AN (C) AND AN (S) | | | | | | |
| | | C♀xC♂ | C♀xS♂ | S♀xC♂ | S♀xS♂ | | |
| | at Gen 43 | 46 | 14 | 9 | 51 | 0.83 | 0.62±0.07 |

CA, Centroamerican semispecies carrying a sex-linked recessive mutation, white; AN, Andean-South Brazilian carrying an autosomal recessive mutation, yellow; C, control line; S, selected line; $X^2_{♂}$, chi-square tests based on ♂♂; * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 1a. The geographic distribution of the six <u>Drosophila paulistorum</u> semispecies.

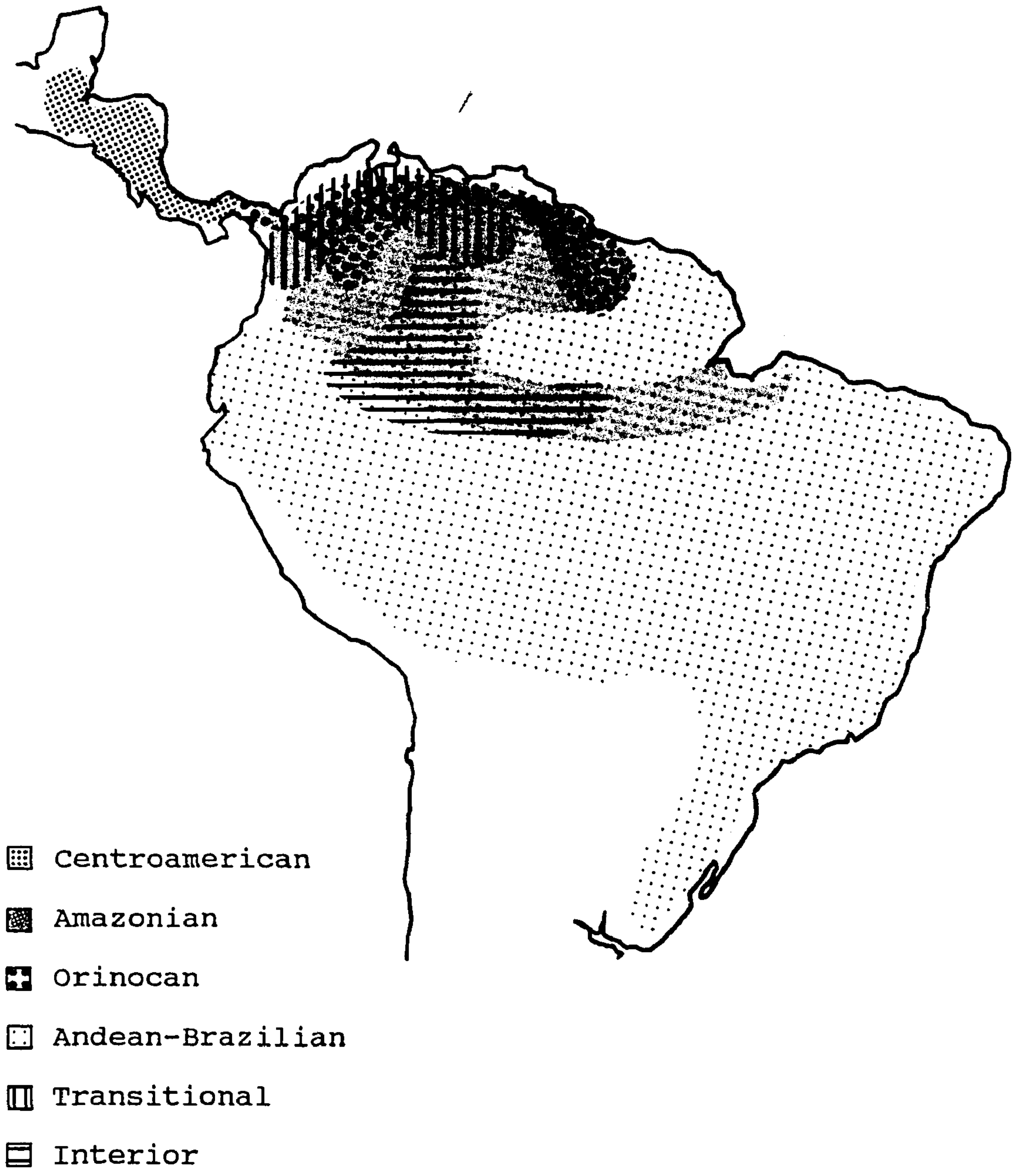

Centroamerican
Amazonian
Orinocan
Andean-Brazilian
Transitional
Interior

Figure 1a

Figure 1b. Cladograms modified from the diagram published in Spassky et al (1971), in which phylogenetic relationships of the sibling species of the Drosophila willistoni group, were based on the study of reproductive affinities, chromosomal differences, sexual behavior, geographic distribution, ecology and morphometric differences. The diagram represents an overall view of phylogenetic braches.

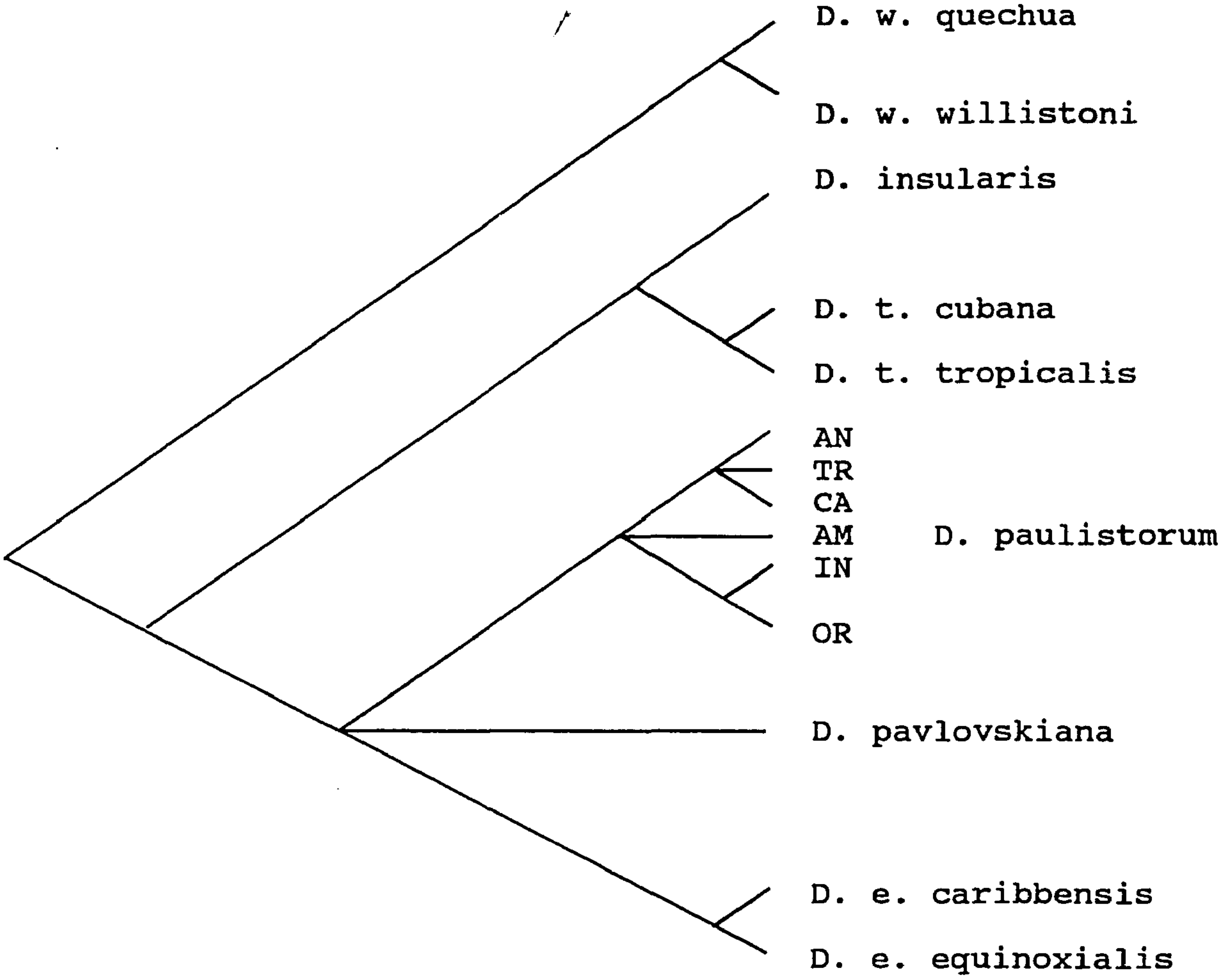

D. w. quechua
D. w. willistoni
D. insularis
D. t. cubana
D. t. tropicalis
AN
TR
CA
AM
IN
OR
D. paulistorum
D. pavlovskiana
D. e. caribbensis
D. e. equinoxialis

Figure 1b

Figure 1c. Phylogeny of species related to Drosophila willistoni, based on electrophoretic differences at 36 gene loci coding for enzymes. The numbers on the branches are estimated nucleotide substitutions per locus that have taken place in evolution. The vertical distances between neighboring taxa are roughly proportional to their genetic differentiation. Seven species in the phylogenetic tree. (from Ayala et al., 1974)

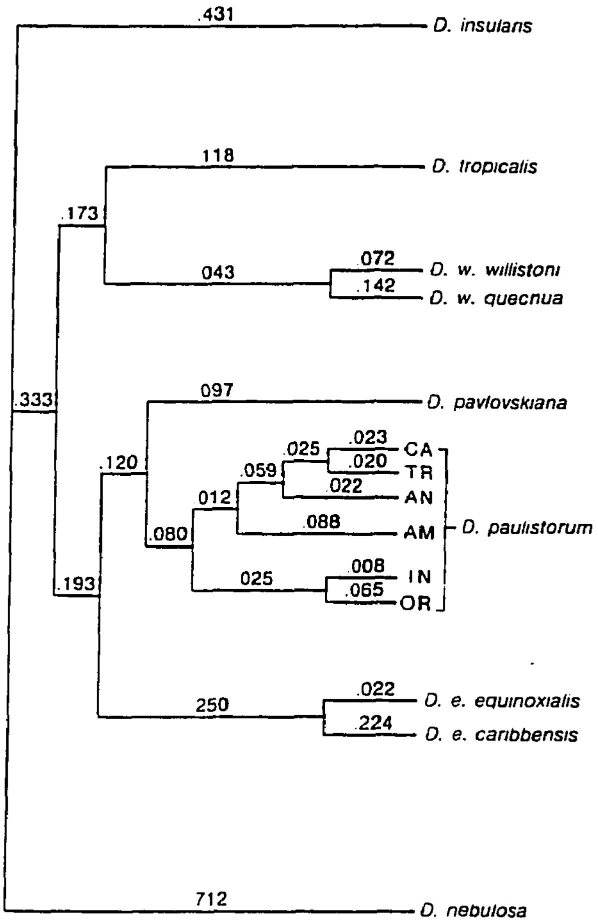

.431
D. insularis
118
.173
D. tropicalis
043
.072
D. w. willistoni
.142
D. w. quechua
.333
097
D. pavlovskiana
.120
.025
.023
CA
.059
.020
TR
012
.022
AN
.080
.088
AM
D. paulistorum
.193
025
.008
IN
.065
OR
.022
D. e. equinoxialis
250
.224
D. e. caribbensis
712
D. nebulosa

Figure Ic

Figure 2. The geographic location of the six strains of Drosophila paulistorum used in experiments: Lancetilla, Honduras (CA); Georgetown, Guyana (OR); Mesitas, Colombia (AN); Santa Marta, Colombia (TR); Belem, Brazil (AM); and Llanos, Colombia (IN).


▲ Centroamerican
▼ Amazonian
■ Orinocan
● Andean-Brazilian
★ Transitional
○ Interior

Figure 2

Figure 3. Comparison of sexual isolation between *Drosophila paulistorum* semispecies: CA, Centroamerican; OR, Orinocan; AM, Amazonian; IN, Interior; AN, Andean-South Brazilian; TR, Transitional.

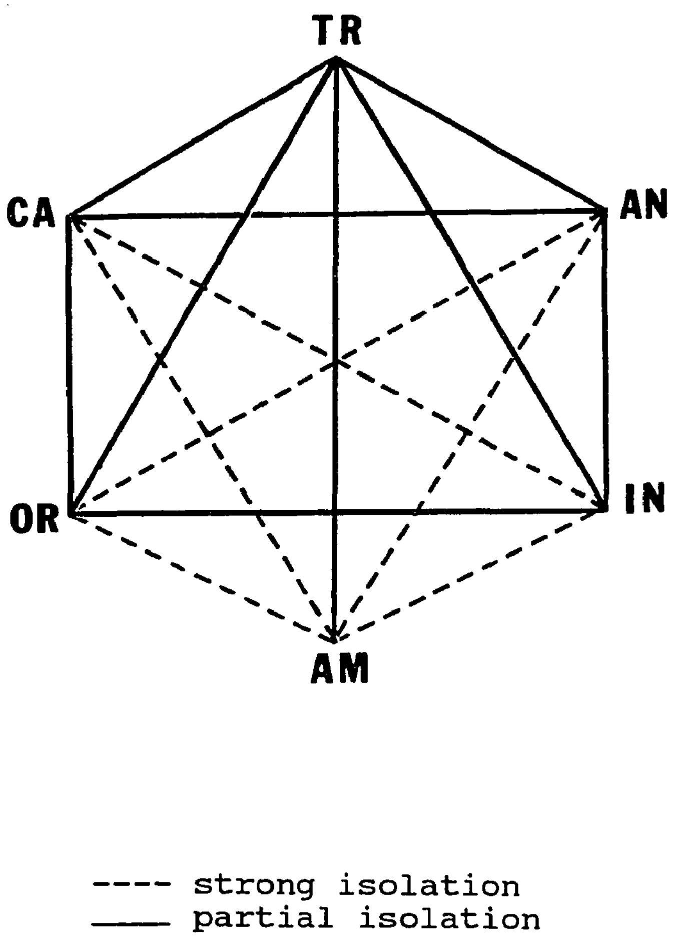


Figure 3

Figure 4. Fritted-glass barrier bridge connecting two *Drosophila paulistorum* semispecies throughout their life cycles.

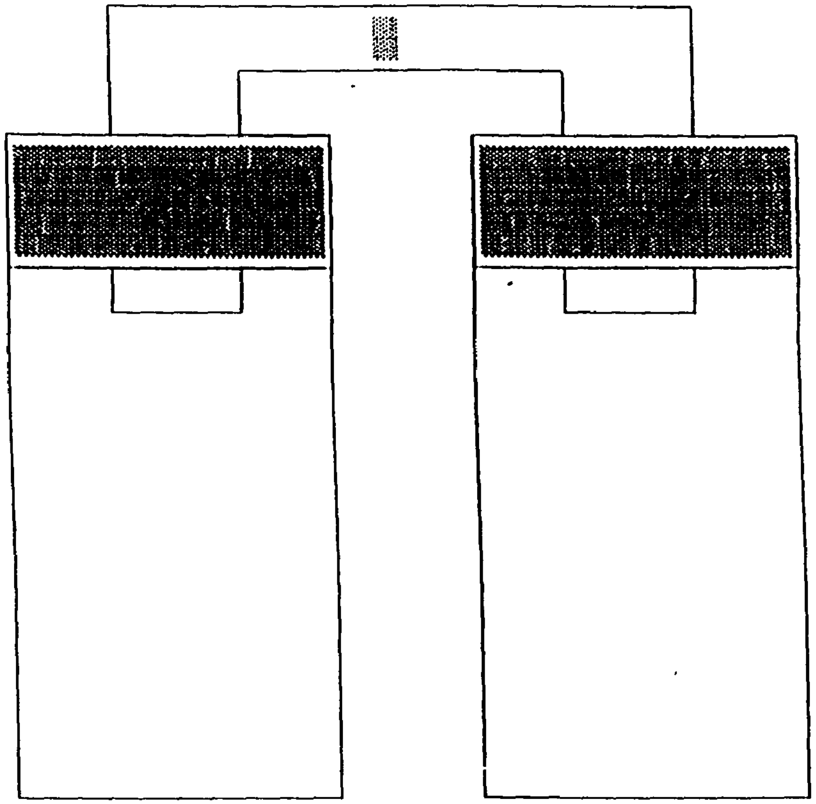


Figure 4

Figure 5. Sexual isolation index versus secluded stage in Drosophila paulistorum semispecies from Honduras (CA) and Guyana (OR). These graphs were based on Table II: a) Subset A1 ($Y = 0.025X + 0.312$, 5 df, $t_s = 11.792$, $p<0.001$); b) Subset A2 ($Y = 0.052X - 0.034$, 5 df, $t_s = 5.939$, $p<0.01$); c) Subset A3 ($Y = 0.047X + 0.049$, 5 df, $t_s = 5.186$, $p<0.01$); d) Subset B1 ($Y = 0.013X + 0.232$, 5 df, $t_s = 5.570$, $p<0.01$); e) Subset B2 ($Y = 0.008X + 0.115$, 5 df, $t_s = 0.793$, ns); and f) Subset B3 ($Y = 0.013X + 0.224$, 5 df, $t_s = 6.725$, $p<0.01$).

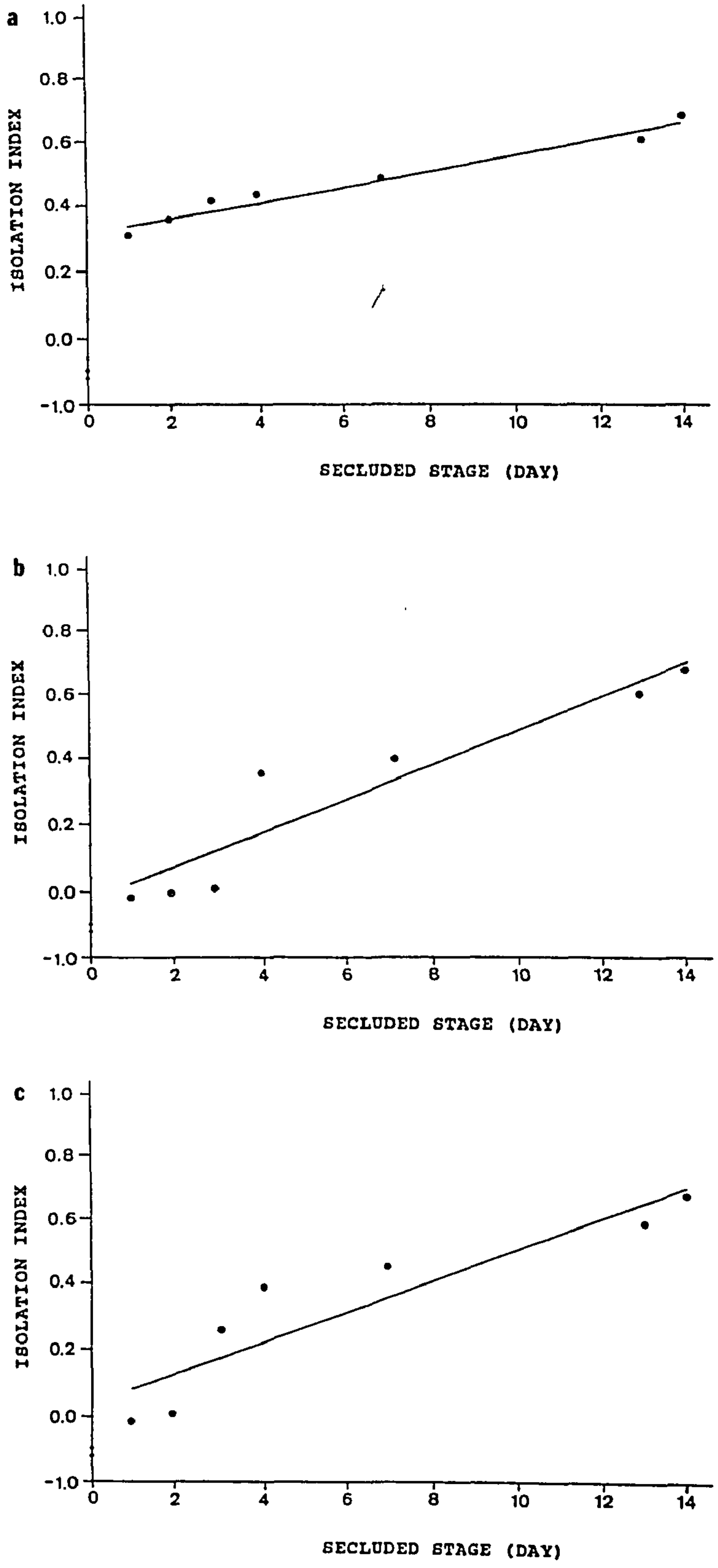

a
b
c
ISOLATION INDEX
1.0
0.8
0.6
0.4
0.2
0.0
-1.0
0
2
4
6
8
10
12
14
SECLUDED STAGE (DAY)

Figure 5

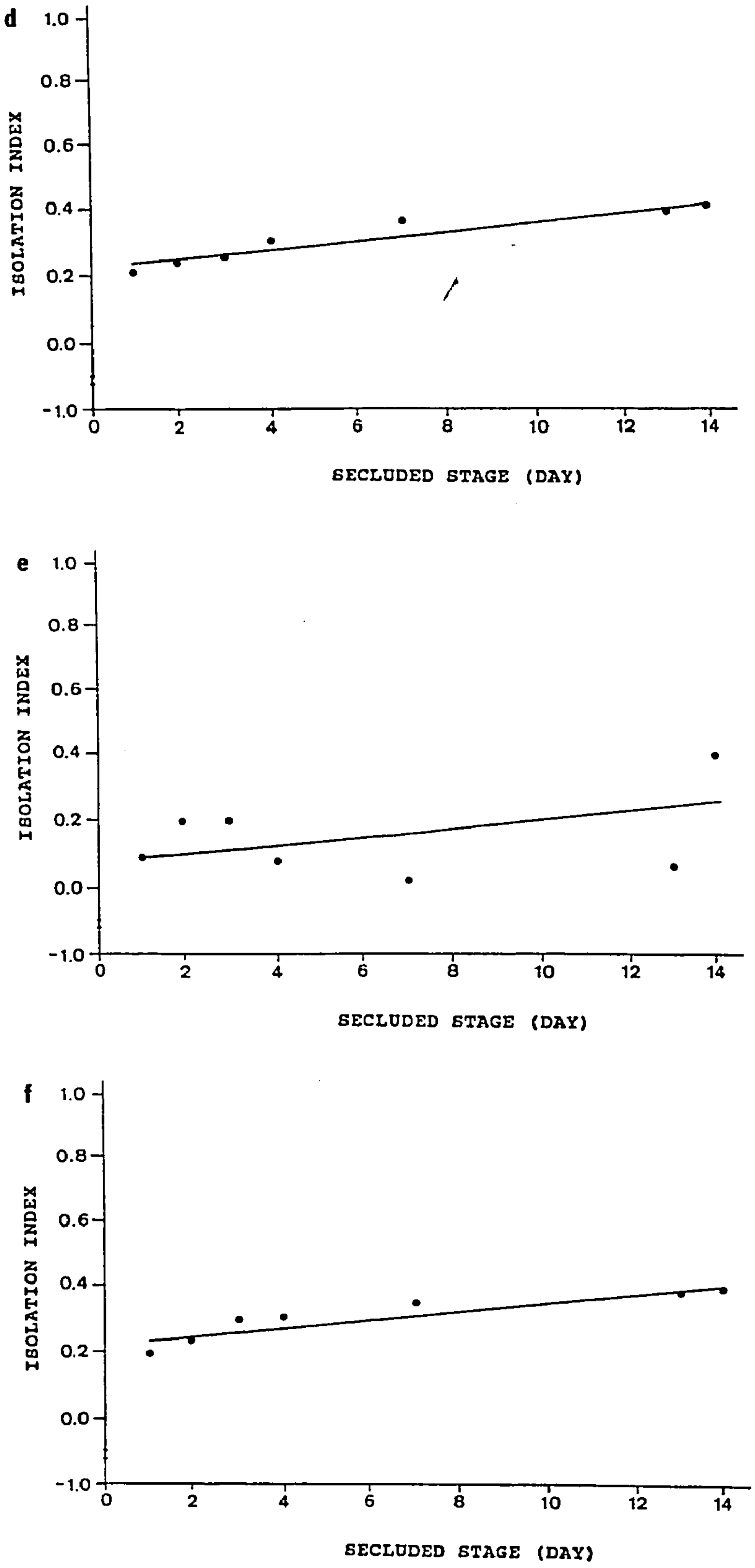


Figure 5 (continued)

Figure 6. Sexual isolation index versus secluded stage in Drosophila paulistorum semispecies from Mesitas, Colombia (AN) and Santa Marta, Colombia (TR). These graphs were based on Table III: a) Subset A1 ($Y = 0.038X + 0.483$, 5 df, $t_s = 3.855$, $p<0.05$); b) Subset A2 ($Y = 0.038X + 0.475$, 5 df, $t_s = 3.655$, $p<0.05$); c) Subset A3 ($Y = 0.038X + 0.471$, 5 df, $t_s = 3.628$, $p<0.05$); d) Subset B1 ($Y = 0.005X + 0.457$, 5 df, $t_s = 1.539$, ns); e) Subset B2 ($Y = 0.018X + 0.289$, 5 df, $t_s = 3.805$, $p<0.05$); and f) Subset B3 ($Y = 0.014X + 0.327$, 5 df, $t_s = 3.542$, $p<0.05$).

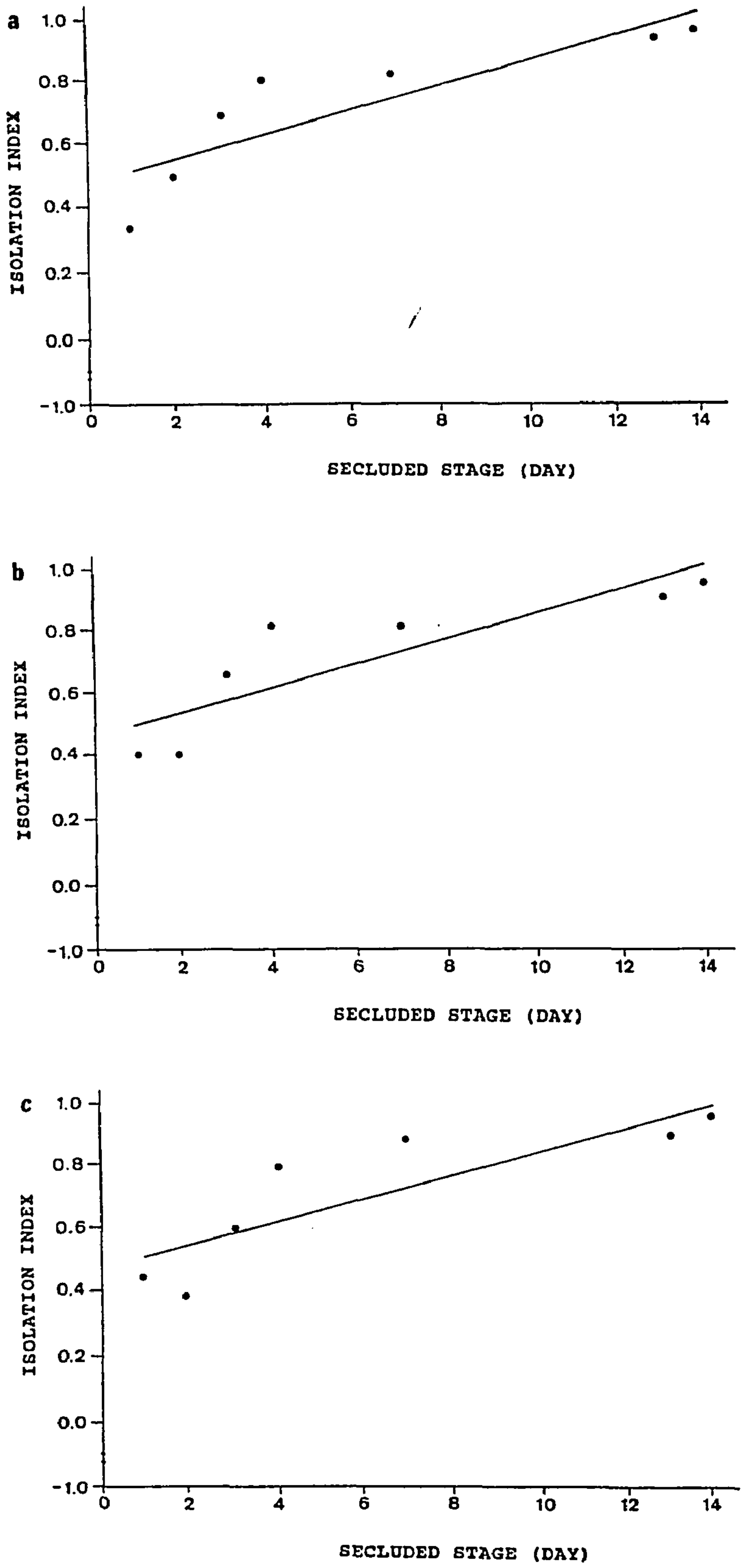

a
b
c
ISOLATION INDEX
1.0
0.8
0.6
0.4
0.2
0.0
-1.0
0
2
4
6
8
10
12
14
SECLUDED STAGE (DAY)

Figure 6

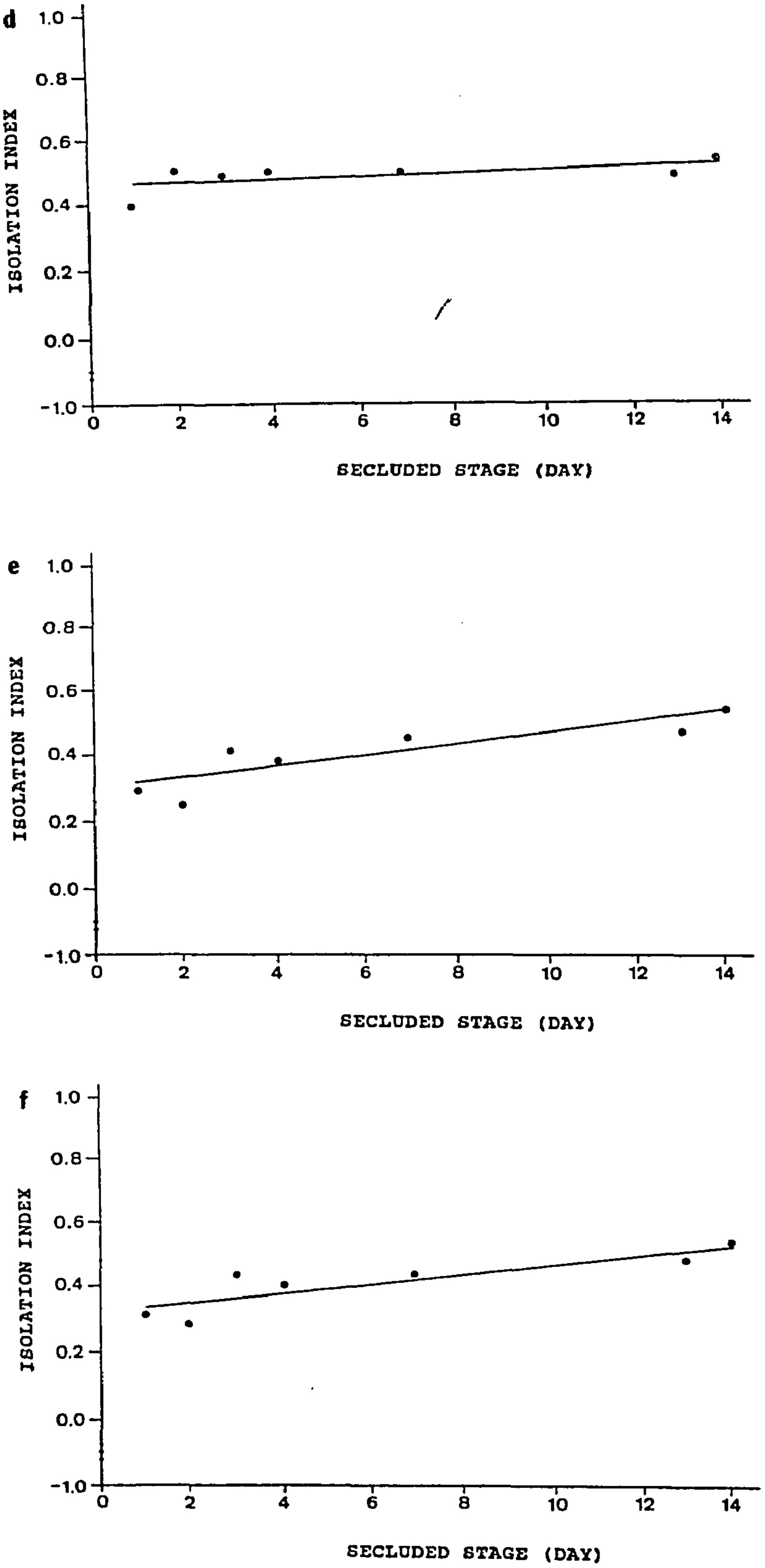


Figure 6 (continued)

Figure 7. Sexual isolation index versus secluded stage in Drosophila paulistorum semispecies from Brazil (AM) and Colombia (IN). These graphs were based on Table IV: a) Subset A1 ($Y = 0.032X + 0.479$, 5 df, $t_s = 4.246$, $p<0.01$); b) Subset A2 ($Y = 0.026X + 0.592$, 5 df, $t_s = 6.053$, $p<0.01$); c) Subset A3 ($Y = 0.02X + 0.686$, 5 df, $t_s = 7.015$, $p<0.001$); d) Subset B1 ($Y = 0.024X + 0.686$, 5 df, $t_s = 2.332$, ns); e) Subset B2 ($Y = 0.007X + 0.891$, 5 df, $t_s = 5.220$, $p<0.01$); and f) Subset B3 ($Y = 0.006X + 0.886$, 5 df, $t_s = 3.890$, $p<0.05$).

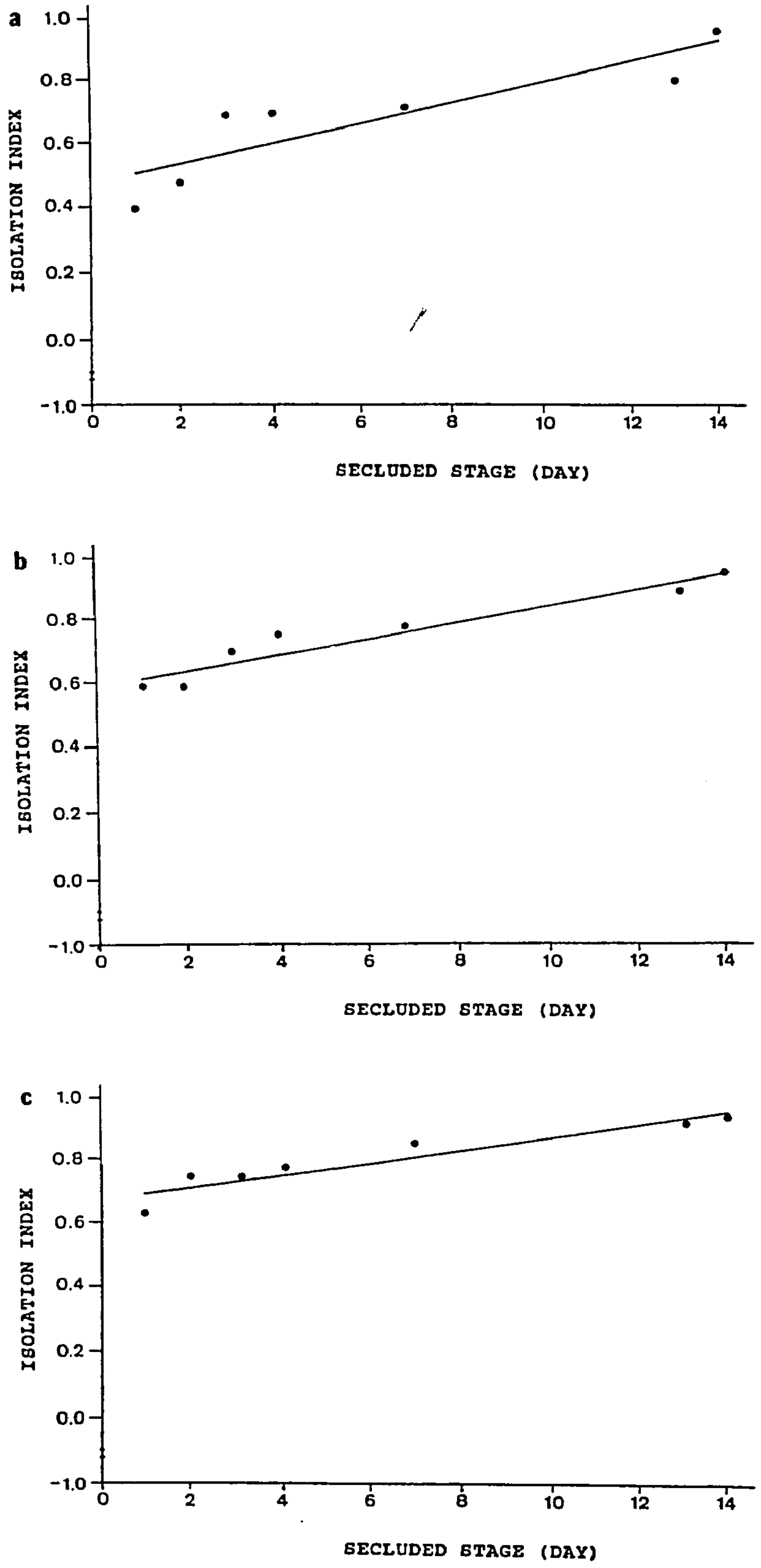

a
b
c
ISOLATION INDEX
1.0
0.8
0.6
0.4
0.2
0.0
-1.0
0
2
4
6
8
10
12
14
SECLUDED STAGE (DAY)

Figure 7

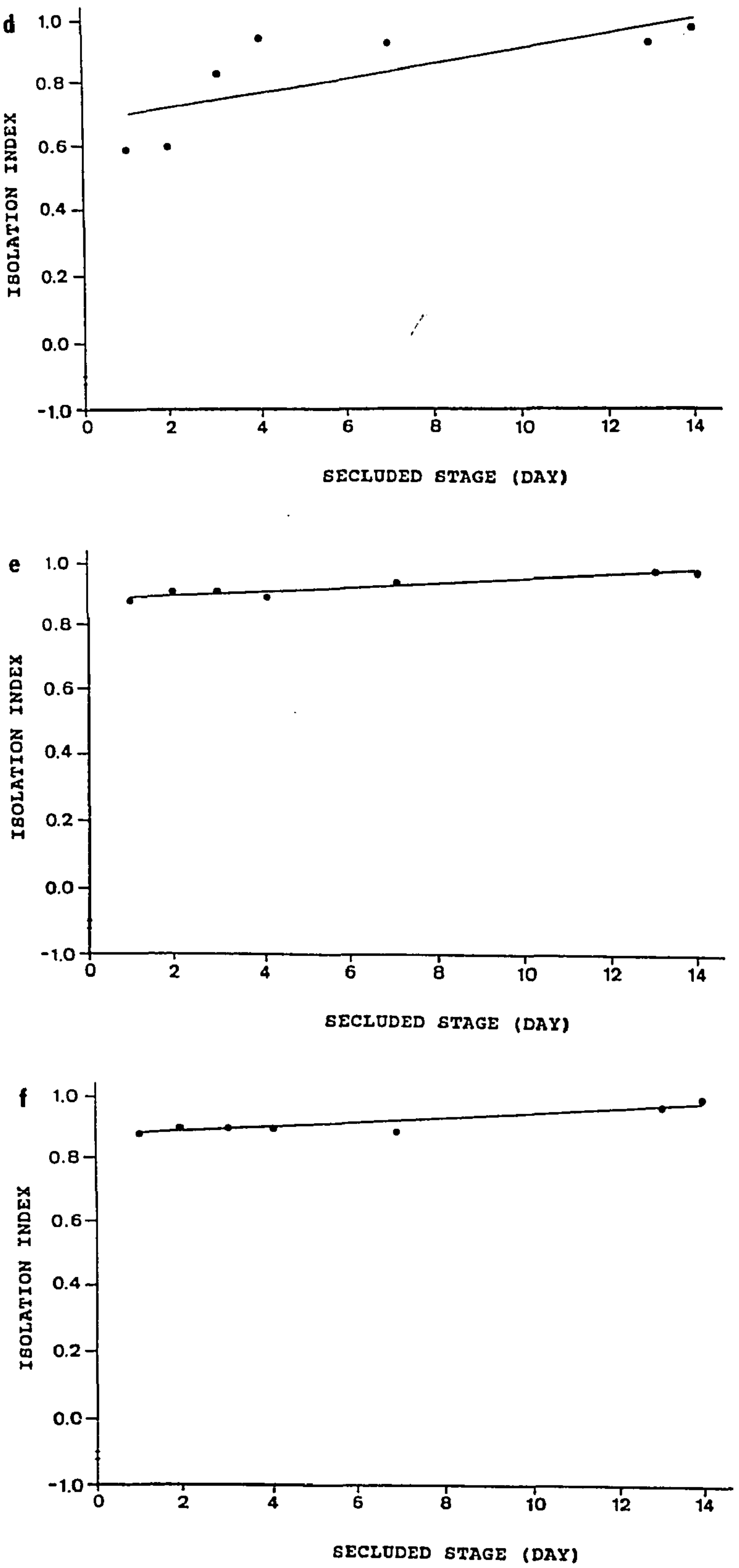
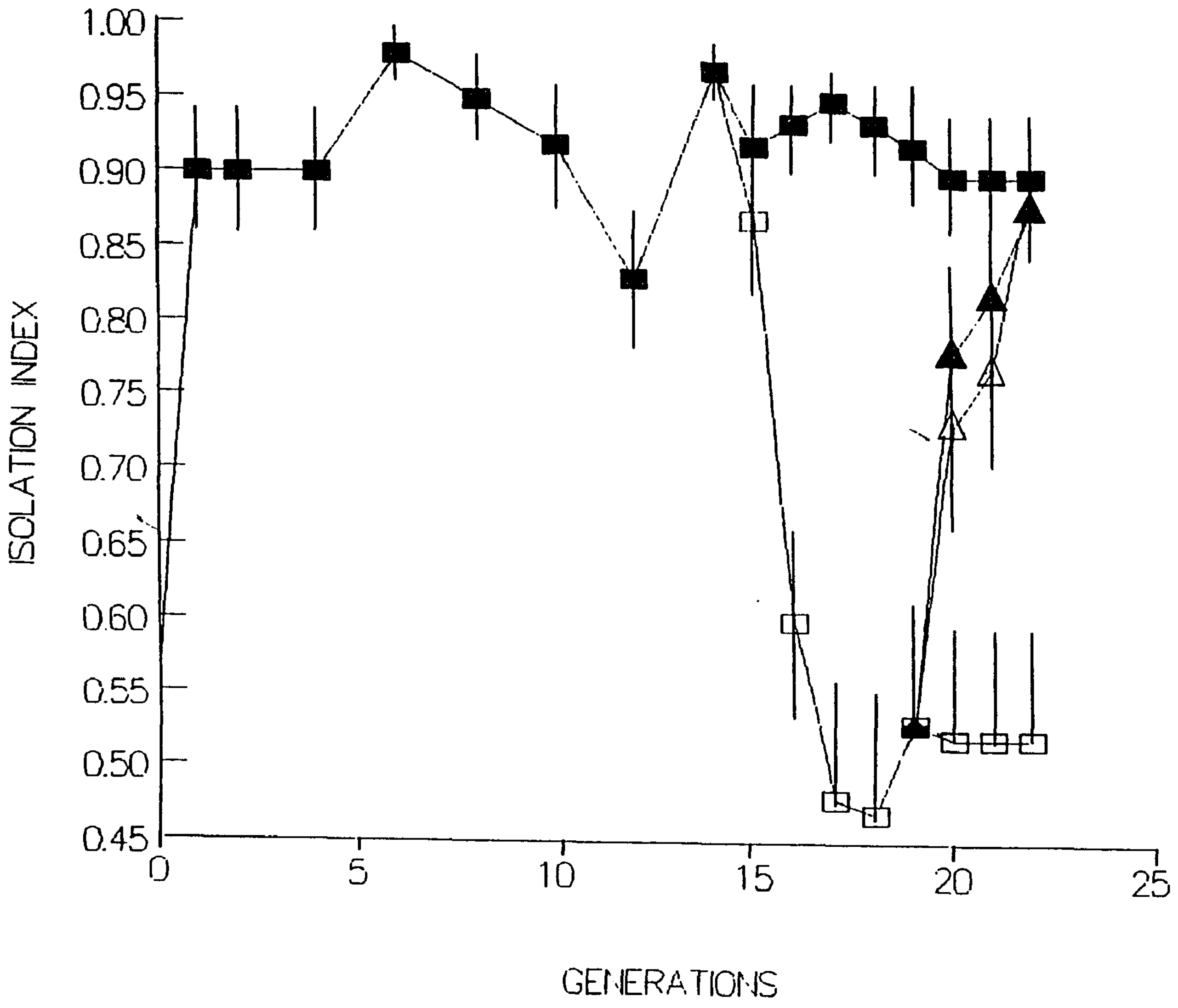


Figure 7 (continued)

Figure 8. Mating responses to different forms of artificial selection in *Drosophila paulistorum* as itemized in Table VIII. ■ = Selected; □ = Relaxed; ▲ = Reselected-1; △ = Reselected-2. Sexual isolation indices reange from -1.00 (heterogamy) through zero (panmixia) to 1.00 (homogamy)



1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
ISOLATION INDEX
0
5
10
15
20
25
GENERATIONS

Figure 8

# APPENDIX 1

The current list of <u>Drosophila</u> <u>paulistorum</u> strains is as follows:

| Semispecies | Code | Collection Site | Number of Parental Lines Merged into One Stock |
|---|---|---|---|
| Amazoniam | | <u>Brazil</u> | |
| | A-28 | Belem | 178 |
| Andean-South | | <u>Colombia</u> | |
| Brazilian | AB-3 | Cucuta | 3 |
| | | <u>Brazil</u> | |
| | AB-comb | Sao Paulo | 1 |
| | | Ceara | 13 |
| | | Xingu | 12 |
| | | Belem | 1 |
| | | <u>Colombia</u> | |
| | AB | Mesitas | 1 |
| | AB-163 | Mesitas | prune eye |
| | AB-165 | Mesitas | yellow body |
| CentroAmerican | | <u>Guatemala</u> | |
| | C-1 | Tikal | 1 |
| | | <u>Honduras</u> | |
| | C-2 | Lancetilla | 1 |
| | C-121 | Lancetilla | white eye |
| Interior | | <u>Colombia</u> | |
| | I-1 | New Llanos | 1 |
| | I-comb | Valparaiso | 54 |
| | | Mitu | 58 |
| | | Leticia | 49 |
| | | Leticia | 31 |
| | I-168 | New Llanos | white eye |

| | | | |
|---|---|---|---|
| Orinocan | | *Guyana* | |
| | O-11 | Georgetown | 15 |
| Transitional | | *Colombia* | |
| | T-1 | Santa Marta | 2 |
| | | *Venezuela* | |
| | T-7 | Guatopa | 7 |
| Carmody<br>(paulistorum-like) | K-2s | *Colombia* | |
| *D. pavlovskiana* | | *Guyana* | |
| | P-1 | Georgetown | 1 |

## APPENDIX 2

Suppose that 1) there are six kinds of female seclusion and one control for a given semispecies, i.e., Centroamerican; 2) from the seclusion experiment, twenty-five females, twelve homogamic males and twelve heterogamic males per combination are placed in an observation chamber; 3) numbers of homogamic matings and those of heterogamic matings are counted. Isolation indices are calculated. The indices are variates in one-way ANOVA and there are four replicates per combination.

| | Group (a = 7) | | | | | | |
|---|---|---|---|---|---|---|---|
| | egg | 1st instar | 2nd instar | 3rd instar | pupa | adult | control |
| 1 | 0.44 | 0.84 | 0.84 | 0.60 | 0.68 | 0.92 | 0.36 |
| 2 | 0.36 | 0.20 | 0.36 | 0.68 | 0.68 | 0.68 | 0.36 |
| 3 | 0.36 | 0.36 | 0.44 | 0.20 | 0.28 | 0.44 | 1.00 |
| 4 | 0.04 | 0.04 | 0.04 | 0.28 | 0.28 | 0.44 | 1.00 |
| I | 0.30 | 0.36 | 0.42 | 0.44 | 0.48 | 0.62 | 0.68 |

<u>Results</u>

1) ANOVA Table

| Source of Variation | df | SS | MS | F | |
|---|---|---|---|---|---|
| Among groups | 6 | 0.444 | 0.074 | 0.930 | ns |
| Control vs. seclusion | 1 | 0.203 | 0.203 | 2.549 | ns |
| Among seclusion | 5 | 0.241 | 0.048 | 0.606 | ns |
| Within groups (Error) | 21 | 1.672 | 0.079 | | |
| Total | 27 | 2.116 | | | |

$F_{0.05[6,21]} = 2.57$; $F_{0.05[1,21]} = 4.32$; $F_{0.05[5,21]} = 2.68$

MSR (Minimum Significant Range) = 0.65 (5%)

2) ANOVA Table with Regression

Each seclusion stage is assigned with day (aging) at which seclusion is initiated; egg (1), 1st instar (2), 2nd instar (3), pupa (7), adult (13), and control (14). Therefore, relationships between secluded stage (day) and isolation index are analyzed.

| Source of Variation | df | SS | MS | F | |
|---|---|---|---|---|---|
| Among groups | 6 | 0.444 | 0.074 | 0.930 | ns |
| Linear Regression | 1 | 0.429 | 0.429 | 139.058 | $p \leq 0.001$ |
| Deviation from regression | 5 | 0.015 | 0.003 | 0.039 | ns |
| Within groups | 21 | 1.672 | 0.079 | | |
| Total | 27 | 2.116 | | | |

$F_{0.05[1,5]}=$ 6.61; $F_{0.01[1,5]}=$ 16.3; $F_{0.001[1.5]}=$ 47.2

3) Homogeneity of slopes (regression coefficients)

Seclusion is conducted in three different ways within a semispecies: 1) females only; 2) males only; and 3) females and homogamic males. The same statistical analyses are applied to 2) & 3). Three regression coefficients are calculated as below.

| | CA | | |
|---|---|---|---|
| | ♀♀ | ♂♂ | ♀♀ + ♂♂ |
| b (reg. coeff.) | 0.025 | 0.052 | 0.047 |

Equality of slopes is tested and results are shown in the form of an anova table.

| Source of Variation | df | SS | MS | F | |
|---|---|---|---|---|---|
| Variation among regressions | 2 | 0.264 | 0.132 | 3.667 | ns |
| Average variation within regressions | 15 | 0.542 | 0.036 | | |

$F_{0.05[2,15]}=$ 3.68; MSD = 0.027 (5%)

Conclusions

1) There are no significant differences among groups, one control and six experimentals.

2) Presence of significant linear regression is observed.

3) CA does differ in response to seclusion depending on sex.

See Sokal and Rohlf (1981) for detail.

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