*Genetica* **91**: 143-149, 1993.


# The effect of superoxide dismutase alleles on aging in *Drosophila*

Robert H. Tyler, †, Hardip Brar, Meena Singh, Amparo Latorre[1], Joseph L. Graves[2], Laurence D. Mueller, Michael R. Rose & Francisco J. Ayala
*Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92717, USA*
[1] *Present address: Departamento de Genetica, Facultad de Biologia, Universidad de Valencia, Spain*
[2] *Author for correspondence*

Received and accepted 22 June 1993

*Key words:* superoxide dismutase, aging, *Drosophila*, evolutionary genetics, senescence

**Abstract**

The effects of superoxide dismutase on aging were tested using two differt experimental approaches. In the first, replicated populations with postponed aging were compared with their controls for frequencies of electrophoretic alleles at the SOD locus. Populations with postponed aging had consistently greater frequencies of the allele coding for more active SOD protein. This allele was not part of a segregating inversion polymorphism. The second experimental approach was the extraction of *SOD* alleles from different natural populations followed by the construction of different *SOD* genotypes on hybrid genetic backgrounds. This procedure did not uncover any statistical effect of *SOD* genotype on longevity or fecundity. There were large effects on longevity and fecundity due to the family from which a particular *SOD* genotype was derived. To detect the effects of *SOD* genotypes on longevity with high probability would require a ten-fold increase in the number of families used.

## Introduction

Studies of the temporal correlates of the aging process do not necessarily indicate the causal mechanisms controlling it, while treatments that shorten life span may kill as a result of novel pathologies (Maynard Smith, 1966; Rose, 1991). For this reason, other experimental strategies are required to unravel the mechanisms that normally control aging. Postponed aging arising from selection for survival to, and reproduction at, later ages requires genetic mitigation of normal aging mechanisms, and therefore constitutes a model system of choice for the analysis of the genetics of aging (Hutchinson & Rose, 1987).

Choice of a good model system for the genetics of aging has not, however, led to immediate breakthroughs in causal understanding. Some electrophoretic studies of the genetic basis of selectively postponed aging in *Drosophila melanogaster* have already been performed (Luckinbill *et al.*, 1989). Some significant correlations between postponed aging and the loci affecting energetic metabolism were found by Luckinbill *et al.*, though that work suffered from a lack of replication. Only one or two selected populations were compared to their control populations. In addition, the founding population sizes were fairly small. Fortunately, these problems are easily remedied using the larger set of stocks created by Rose (e.g. 1984), in which there are five selected lines and five controls, the entire set of stocks coming from a large wild-caught sample of *D. melanogaster* from the endemic Ives population of South Amherst, Massachusetts.

A more profound problem with electrophoretic comparisons of postponed-aging stocks with their controls is that of linkage disequilibrium. When postponed-aging stocks are created by selection from a common founding population, the particular linkage disequilibria of that population will be in

common among all derivatives undergoing selection. Allelic state correlations between physically proximal loci will be maintained for some part of the selection process, causing nearby loci to undergo parallel changes of allele frequency in all selected stocks. Therefore, stocks with selectively postponed aging are not necessarily, by themselves, reliable systems for inferring the causal involvement of particualar loci in aging.

One potential solution to this difficulty is to use stocks with postponed aging to identify initial candidate genes, genes that might be involved in postponed aging, or that might change in allelic composition because of linkage disequilibrium alone. Then genotypes at this locus can be arbitrarily assembled from different natural populations, and tested for their effects upon aging (cf. Serradilla & Ayala, 1983). In this way, the problem of the original linkage relations in the founding population before selection can be overcome. In the present paper, we apply this approach to one locus of great interest to gerontologists, that coding for Cu, Zn superoxide dismutase. This free radical scavenging molecule is of interest because of its role in catalyzing the conversion of superoxide radicals to hydrogen peroxide, the latter then undergoing conversion to water due to the action of catalase (McCord & Fridovich, 1969). Since superoxide radicals are highly damaging for macromolecules such as protein, lipid, and nucleic acid, the cumulative effects of such free radicals have long been proposed as a major factor in aging (e.g. Harman, 1956). For this reason, we were particularly interested in applying the combination of stocks with postponed aging and sampled alleles from wild populations to test for the effects of superoxide dismutase alleles upon aging. In natural populations of *D. melanogaster*, Cu, Zn superoxide dismutase has *F* ('fast') and *S* ('slow') allelomorphs, with *S* being rarer but of greater *in vitro* activity (Lee, Misra & Ayala, 1981). On the free radical theory of aging, this allelomorph should have elevated frequencies in populations that are longer-lived. In addition, the *S* allele should give rise to increased longevity, on average, in flies of *SS* or *FS* genotype, relative to *FF* genotypes. The present article tests these predictions. We find that some of these predictions are borne out, but others are not.

## Materials and methods

*Postponed aging stocks*

The postponed aging populations used in the present study were derived from a long-standing wild population that had been sampled in 1975 (Rose, 1984). All fly culture proceeded at 25 °C with abundant food, little crowding, and moderate humidity. In Feb. 1980, one generation of the base population was used to found ten outbred populations, five of which (called $B_1$-$B_5$) were kept under the same conditions as the base population, with two-week discrete generations. The other five were cultured using females of increasingly greater ages as the generations progressed, such that by late 1981 all reproducing females had to attain 70 days of age (Rose, 1984). Such populations are designated $O_1$-$O_5$, with no correspondence between B and O subscripts. Since 1981, cohorts sampled from the O populations have had significantly greater mean and maximum longevities, relative to B populations (Rose, 1984; Hutchinson & Rose, 1991).

In addition to this longevity difference, B and O populations have a number of other differences which indicate that O flies live longer because of postponed aging. These differences include increases in: tethered flight duration (Graves & Rose, 1990), spontaneous locomotion at later ages (Service, 1987), 24-h egg-laying at later ages (Rose, 1984), and resistance to stress (Service *et al.*, 1985). These findings strongly suggest that the longer-lived O populations indeed possess postponed aging, through both general and late-age enhancements in physiological functions. Derivatives of the O populations have also been subjected to relaxed selection (R populations), in which cultivation returned to 2-week generations (Service, Hutchinson & Rose, 1988). The phenotypes of these R populations have largely returned to those of the control B populations (Graves *et al.*, 1992; and unpub. data), including mean longevity. If superoxide dismutase is a causal factor postponing aging, then the electrophoretic profile of the R populations should be identical to that of the B populations.

*Extraction of SOD genotypes from natural populations*

*D. melanogaster* females were collected from Culver City, Los Angeles County, in southern Califor-

nia and El Rio Vineyard, San Joaquin Country, in northern California. From each population, 20 isofemale lines were made homozygous for each of both the *F* and *S* superoxide dismutase alleles. This was done by inbreeding and electrophoretic screening over multiple generations.

These inbred lines were then crossed with each other in pairs to create lines of known SOD genotype but hybrid background. This procedure mitigates problems of inbreeding depression. Such crosses were performed by crossing in each case 20 males from one line with 20 females from a different line. Four different genotypes were constructed: *FF, SS, FS,* and *SF*, where the latter pair of genotypes differ according to the maternal genotype.

*Assay procedures*

All electrophoretic procedures used the methods described in Ayala *et al.* (1972). Polytene chromosomes were extracted from larvae of B and O populations and stained. Surveys were made of 29-55 different chromosomes for the presence of inversion heterozygotes on each arm. All assays of life-history phenotypes used control-density larval rearing, with 25-50 larvae per vial. Adult females were kept together with two males, in vials of banana-molasses food for the Culver City samples and corn meal-flour food for the El Rio samples. Fecundity assays were made periodically, using a charcoal-colored high-agar medium without yeast. Vials were examined each day for survival of the adults.

In these experiments there were block effects which confounded two sources of experimental variation. The first of these is the population of origin and the second is laboratory handling. The assays for each population were conducted in different laboratories. Thus, different personnel, incubators and type of food were used for collecting data from each population. Consequently, all statistical analyses treated results from El Rio and Culver City as two blocks.

*Table 1.* Frequency of the S('slow') allele in B and O populations for Cu,Zn superoxide dismutase (SOD). Only two electromorphs were detected. Electrophoresis was performed according to Ayala *et al.* (1972); *n* is the number of individuals sampled.

| Line | n | *FF* | *FS* | *SS* | *S*Frequency |
|---|---|---|---|---|---|
| $B_1$ | 50 | 50 | 0 | 0 | 0.00 |
| $B_2$ | 48 | 50 | 0 | 0 | 0.00 |
| $B_3$ | 50 | 50 | 0 | 0 | 0.00 |
| $B_4$ | 24 | 24 | 0 | 0 | 0.00 |
| $B_5$ | 24 | 24 | 0 | 0 | 0.00 |
| Average allele frequency | | | | | 0.00 |
| Line | n | *FF* | *FS* | *SS* | *S*Frequency |
| O1 | 48 | 26 | 17 | 5 | 0.28 |
| O2 | 48 | 39 | 8 | 1 | 0.10 |
| O3 | 48 | 22 | 15 | 11 | 0.39 |
| O4 | 49 | 20 | 26 | 3 | 0.33 |
| O5 | 48 | 36 | 9 | 3 | 0.16 |
| Average allele frequency standard error | | | | | 0.25 ± 0.05 |

## Results

*Allele frequencies in postponed-aging stocks*

As shown in Table 1, the *S* allele is significantly more frequent in O populations, averaging about 25%, with no *S* alleles detected in the B population samples. Indeed, this average frequency of *S* in the O populations is greater than has been observed before for this allele in natural populations of *Drosophila* (Smit-McBride, Moya & Ayala, 1988; Singh, Hickey & David, 1982 and unpub. data). While these samples suggest that the B populations entirely lack the *S* allele, further sampling of these populations has produced a few instances where flies from B populations carry the *S* allele heterozygously. The R populations have the same profile as the B sample shown here, the *S* allele being absent. All of these findings are in accord with the free-radical theory of aging, in that the more active allele is significantly more frequent in populations with postponed aging.

But the conclusion that SOD is causally related to aging could be erroneous if the *S* allele is tightly linked to an allele which itself postpones aging. The total number of generations separating B from O populations is over 300, the two population-types have been replicated five-fold, and the census population sizes have been in the thousands. These experimental design features facilitate attainment of linkage equilibrium at unselected loci. Nonetheless, if the *S* allele were associated with an inversion polymorphism, recombination could be too weak to prevent linkage disequilibrium between the *S* allele and a selected allele at another locus within the same inversion. Table 2 shows the results from

*Table 2.* Number of polytene chromosome arms sampled and inversions observed for B and O populations.

| | Chromosome arm | | | | | |
|---|---|---|---|---|---|---|
| Line | X | 2R | 2L | 3R | 3L | Inversions |
| $B_1$ | 38 | 48 | 48 | 48 | 48 | None |
| $B_2$ | 30 | 41 | 41 | 41 | 41 | None |
| $B_3$ | 30 | 41 | 41 | 41 | 41 | None |
| $B_4$ | 36 | 50 | 50 | 50 | 50 | None |
| $B_5$ | 36 | 48 | 48 | 48 | 48 | None |
| $O_1$ | 35 | 49 | 49 | 49 | 49 | None |
| $O_2$ | 28 | 44 | 44 | 44 | 44 | None |
| $O_3$ | 29 | 40 | 40 | 40 | 40 | None |
| $O_4$ | 36 | 52 | 52 | 52 | 52 | One on 2R |
| $O_5$ | 40 | 55 | 55 | 55 | 55 | None |

a study of the cytology of 2205 polytene chromosomes. Only one inversion variant was detected (In(2R)NS), on the right arm of chromosome 2 in the $O_4$ population. Moreover, the superoxide dismutase locus is located on chromosome 3, in any case. Therefore, the only remaining countervailing hypothesis would be linkage disequilibrium with a favored allele that is physically very close to SOD on chromosome 3. Any such allele is unlikely to have the same linkage relationship with SOD in the unrelated California populations of *D. melanogaster* discussed below.

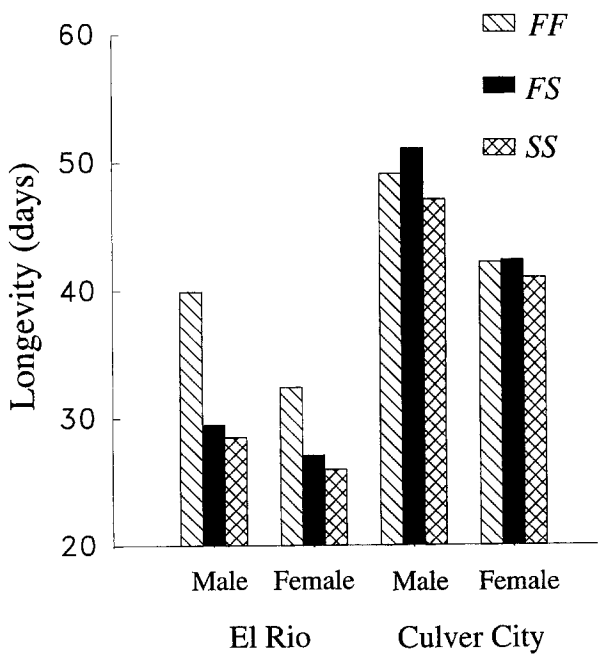


*Fig. 1.* The mean longevity for each SOD genotype as a function of sex and population.

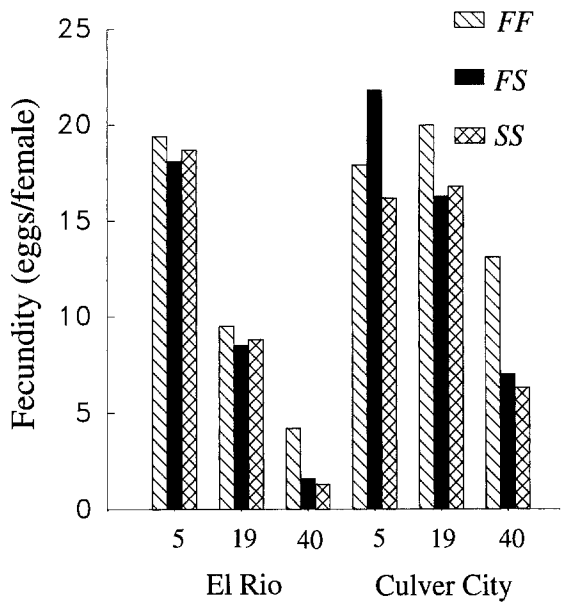


*Fig. 2.* Female fecundity for each SOD genotype at three different ages 5, 19 and 40 days in the El Rio and Culver City populations.

*Effects of SOD genotype on aging phenotypes*

The results of analyses of variance for longevity and age-specific fecundity are shown in Tables 3

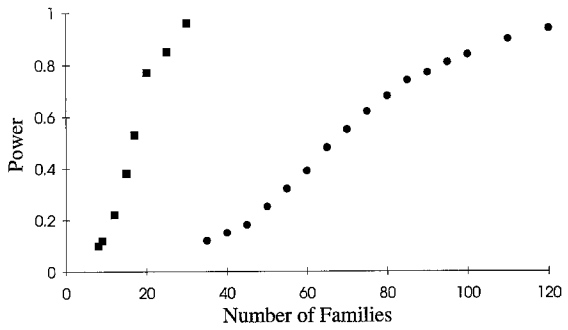


*Fig. 3.* The power of detecting significant genotype effects on longevity (circles) and fecundity (squares) as a function of the number of families sampled within each genotype. These calculations utilized the longevity data from the El Rio population to estimate the differences in longevity between genotypes and the variance between families and the fecundity data at day 40 from Culver City.

*Table 3.* Results of the analysis of variance (ANOVA) on longevity. Population was treated as a block effect.

| Source of variation | Sum of squares | Degrees of freedom | Mean square | F | Significance |
|---|---|---|---|---|---|
| Population | 139,030 | 1 | 139,030 | | |
| Genotype | 1,697 | 2 | 849 | 0.255 | NS |
| Sex | 25,418 | 1 | 25,418 | 80 | <0.001 |
| Family (Genotype) | 56,475 | 17 | 3,322 | 10.5 | <0.001 |
| Sex by Genotype | 219 | 2 | 109 | 0.03 | NS |
| Error | 907,114 | 2,867 | 316 | | |

NS = not significant

and 4 respectively. Throughout these analyses, it is apparent that there is little significant effect of genotype upon aging patterns (Figs. 1-2). To the extent to which there is a directional effect, the *F* allele is associated with increased longevity, although not significantly so.

In these experiments within each genotypic class were nested several families (5-10). In every case (Tables 3-4) there was a statistically significant effect of families on either longevity or fecundity. Genotypic effects were assessed by placing the family mean square in the denominator of the F ratio. Consequently, it appears that the number of families sampled will have a great impact on the statistical power of these tests. We should add that ANOVAs on the separate experimental results from El Rio or Culver City do not reveal significant effects of genotype due to the large between-family variation.

In Figure 3 the statistical power of the longevity assay on the El Rio population and the day 40 fecundity assay on the Culver City population are shown. These particular examples were chosen since they show the greatest differences between genotypes. For instance, the power of the ANOVAs on fecundity at day 5 and 19 is still below 20%, even when 100 families are sampled within each genotype.

These results suggest that the genetic background that is unique to each family is causing variation that obscures any differences that the SOD locus might produce. Except for the possibility of late fecundity, samples of about 100 families per genotype would be required to insure a high

*Table 4.* Results of the analysis of variance (ANOVA) on fecundity. The ANOVAs were carried out on fecundity at day 5, 19 and 40 separately. Population was treated as a block effect.

| Source of variation | Sum of squares | Degrees of freedom | Mean square | F | Significance |
|---|---|---|---|---|---|
| | | **Day 5 Fecundity** | | | |
| Population | 347 | 1 | 347 | | |
| Genotype | 12,325 | 2 | 6,163 | 2.76 | NS |
| Family (Genotype) | 37,910 | 17 | 2230 | 10.67 | <0.001 |
| Error | 357,348 | 1,709 | 209 | | |
| | | **Day 19 Fecundity** | | | |
| Population | 26,413 | 1 | 26,413 | | |
| Genotype | 749 | 2 | 374 | 0.428 | NS |
| Family (Genotype) | 14,884 | 17 | 876 | 5.76 | <0.001 |
| Error | 228,493 | 1,503 | 152 | | |
| | | **Day 40 Fecundity** | | | |
| Population | 6,360 | 1 | 6,360 | | |
| Genotype | 1,536 | 2 | 768 | 3.40 | 0.06 |
| Family (Genotype) | 3,842 | 17 | 226 | 2.89 | <0.001 |
| Error | 53,281 | 681 | 78 | | |

NS = not significant

probability of detecting differences between genotypes of the magnitude observed in this study.

## Discussion

The present study finds that more active superoxide dismutase alleles are associated with postponed aging in laboratory stocks. We have failed to demonstrate that such alleles directly increase life span or later fecundity, but this failure arose from a lack of experimental power. That is to say, this negative result is not a pertinent refutation of the free radical mechanism for aging. The lack of inversion polymorphisms involving *SOD* indicates that alleles at the locus are not in linkage disequilibrium with distal loci. However, linkage disequilibrium with physically proximal loci could nevertheless be the factor responsible for the association between superoxide dismutase and postponed aging. Luckinbill *et al.* (1989) found no difference in *SOD* allele frequencies between their long- and short-lived populations of *D. melanogaster*. Luckinbill's study employed two replicates of each treatment, and thus has less statistical power then the present study. Moreover, the frequency of the *S* allele is low in natural populations ($\approx$ 1%), so the *S* allele may have been absent in the original founding sample, or lost by chance during the early generations of selection.

Of great relevance to the present study is the finding of Reveillaud, Niedzwiecki and Fleming (1991), who transformed *D. melanogaster* stocks with highly active bovine SOD DNA to which a strong actin promoter had been linked. In addition to greatly increasing SOD activity levels in transformed flies, adult life span was significantly increased among replicate transformants. Although this increase was of no greater magnitude than 10%, it constitutes a powerful demonstration that more active SOD can give rise to increased lifespan.

Comparing the results with (i) allele frequencies of postponed aging stocks, (ii) alleles extracted from natural populations, and (iii) transformation experiments with extremely active SOD genes (Reveillaud, Niedzwiecki & Fleming, 1991), some conclusions suggest themselves. These disparate results can be reconciled if it is supposed that the *FS SOD* electromorphs have differential effects on aging that are smaller in magnitude than the differential effects between bovine and dipteran *SOD*. If bovine *SOD* transformation can increase life span by at most 10%, relative to dipteran *SOD*, then the effect of the normal dipteran allelic difference might be about 2-4%. The problem for experiments in which alleles are extracted from natural populations is that the hybrid lines constructed from the isofemale inbreds have substantial differences in life span arising from different accidents of fixation in their ancestral inbred lines. Such differences among lines would thus swamp the effect of longevity differences between genotypes at the *SOD* locus, because the latter differences are so small.

From this line of argument, two final inferences can be drawn. Firstly, this paper provides additional evidence supporting the involvement of *SOD* in postponing, and thus controlling, aging in *Drosophila*, particularly in light of the bovine DNA transformation results. Secondly, the extraction of *SOD* alleles from natural populations has been unable to demonstrate a quantitative effect of this locus on aging phenotypes due to variation in genetic backgrounds between families. Transformation experiments might be preferable in any such tests.

## Acknowledgements

Lisa M. Chiu and Sarah S. Kwak provided considerable technical help in the performance of these experiments. This research was supported in part by grants PHS AG06346 and AG09970 from the National Institute of Aging (USA) to MRR and the Department of Energy (USA) to FJA, and in part by the estate of Robert H. Tyler. We are grateful to the Tyler family and attornies for their support, cooperation, and encouragement.

## References

Ayala, F. J., J. R. Powell, M. L. Tracey, C. A. Mourao & S. Perez-Salas, 1972. Enzyme variability in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *Drosophila willistoni*. Genetics 70: 113-139.

Graves, J. L. & M. R. Rose, 1990. Flight duration in *Drosophila melanogaster* selected for postponed senescence, pp. 59-65 in Genetic effects on aging. II, edited by D. E. Harrison. Telford Press, Caldwell, N.J.

Graves, J. L., E. C. Toolson, C. Jeong, L. N. Vu & M. R. Rose 1992. Desiccation, flight, glycogen, and postponed senescence in *Drosophila melanogaster*. Phys. Zool. 65: 268-286.

Harman, D., 1956. Aging - a theory based on free radical and radiation chemistry. J. Gerontol. 11: 298-300.

Hutchinson, E. W. & M. R. Rose, 1987. Genetics of aging in insects. Rev. Biol. Res. Aging 3: 63-70.

Hutchinson, E. W. & M. R. Rose, 1991. Quantitative genetics of postponed aging in *Drosophila melanogaster*. I. Analysis of outbred populations. Genetics 127: 719-727.

Lee, Y. M., H. P. Misra & F. J. Ayala, 1981. Superoxide dismutase in *Drosophila melanogaster*. Biochemical and structural characterization of allozyme variants. Proc. Natl. Acad. Sci. USA 78: 7052-7055.

Luckinbill, L. S., T. A. Grudzien, S. Rhine & G. Weisman, 1989. The genetic basis of adaptation to selection for longevity in *Drosophila melanogaster*. Evol. Ecology 3: 31-39.

Maynard Smith, J., 1966. Theories of aging, pp. 1-35 in Topics in the Biology of Aging, edited by P. L. Krohn. Interscience, NY.

McCord, J. M. & I. Fridovich, 1969. Superoxide dismutase: An enzymatic function for erythrocuprin. J. Biol. Chem. 224: 6049-6055.

Reveillaud, I., A. Niedzwiecki & J. E. Fleming, 1991. Expression of bovine superoxide dismutase in *Drosophila melanogaster* augments resistance to oxidative stress. Mol. Cell. Biol. 11: 632-40.

Rose, M. R., 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution 38: 1004-1010.

Rose, M. R., 1991. Evolutionary Biology of Aging. Oxford University Press, NY.

Serradilla, J. M. & F. J. Ayala, 1983. Ecological and evolutionary divergence in five species of *Drosophila*. Z. zool. Syst. Evolut.-forsch. 21: 194-200.

Service, P. M., 1987. Physiological mechanisms of increased stress resistance in *Drosophila melanogaster* selected for postponed senescence. Physiol. Zool. 60: 321-326.

Service, P. M., E. W. Hutchinson, M. D. MacKinley & M. R. Rose, 1985. Resistance to environmental stress in *Drosophila melanogaster* selected for postponed senescence. Physiol. Zool. 58: 380-389.

Service, P. M., E. W. Hutchinson & M. R. Rose, 1988. Multiple genetic mechanisms for the evolution of senescence in *Drosophila melanogaster*. Evolution 42: 708-716.

Singh, R. M., D. A. Hickey & J. David, 1982. Genetic differentiation between geographically distant populations of *Drosophila melanogaster*. Genetics 101: 235-256.

Smit-McBride, Z., A. Moya & F. J. Ayala, 1988. Linkage disequilibirium in natural and experimental populations of *Drosophila melanogaster*. Genetics 120: 1043-1051.