Multiple paternity in two natural populations (orchard and vineyard) of *Drosophila*

(fertility/fitness/male mating success/male–female interactions/*Drosophila melanogaster*)

MARIA DOLORES OCHANDO*†, AURELIO REYES†, AND FRANCISCO J. AYALA*‡

*Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525; and †Departamento de Genética, Universidad Complutense, 28040 Madrid, Spain

Contributed by Francisco J. Ayala, August 7, 1996

ABSTRACT Male mating success is an important fitness component in *Drosophila*. The seminal fluid conveyed with the sperm inhibits the proclivity of the female to remate and reduces her fitness. Nevertheless, females may remate before they have exhausted the sperm from the first male and consequently use sperm from both males. We have studied concurrent multiple paternity (CMP) in two *Drosophila melanogaster* populations, from an apple orchard and a vineyard just after harvest. CMP is high in both populations, somewhat greater than 50%; but it is not significantly higher in the vineyard, where the population density is much greater than in the orchard. Population density had been thought to be an important determinant of CMP incidence. We have used four gene loci coding for enzymes as independent markers for detecting CMP.

Herbert Spencer's phrase "survival of the fittest," unwisely adopted by Darwin, is an inadequate descriptor of natural selection for various reasons. Relevant for the present purposes is that it focuses on survival without acknowledging the determinative contribution of the reproductive function. Natural selection results from differential reproduction over the generations, which is measured by the parameter known as "Darwinian fitness," or simply "fitness." Organisms must survive to reproduce, but it is the number of their progeny over the generations that determines whether a gene or trait will persist, whether it will replace others or be obliterated.

The contribution made to natural selection by the biological processes directly related to reproduction is called "fertility" in population genetics (1, 2). The fertility of a genotype has been shown to be an important component of its fitness (2, 3). Two major constituents of fertility are the fecundity of females and the mating success of males.

Drosophila females, like those of many other insects, store the sperm received from a male, which sperm is then gradually released for fertilizing eggs as these mature and are laid. But *Drosophila* females are able to mate again before the sperm from a previous mating has been exhausted, so that competition between sperm from different males may occur inside the female reproductive organs. Moreover, the seminal fluid injected by a male affects, often negatively, the fitness of the inseminated female and also the proclivity of the female to mate again. A sort of tug-of-war between the sexes occurs, because the fitness interests of males and females are partly at odds (3–11).

The incidence of concurrent multiple paternity (CMP) varies from one to another *Drosophila* species (ref. 10, and see also ref. 12; although many thought until a few years ago that it might be a laboratory phenomenon, unlikely to occur in natural conditions), but variation seemingly also occurs among populations of the same species (13–15), a finding that has

been attributed to environmental variables, such as population density (13). In the present paper, we investigate CMP in two natural populations of *Drosophila melanogaster*, from an apple orchard and from a winegrape vineyard shortly after harvest. We use four independent genetic markers to ascertain CMP. Even though the population density in the vineyard is several orders of magnitude greater than in the orchard, the incidence of CMP is not significantly different, about 0.5 in both.

MATERIALS AND METHODS

We collected *Drosophila melanogaster* flies in two natural habitats: (i) an apple orchard, near Davis, California, at the end of August and (ii) a winegrape vineyard in Requena (Valencia), Spain, in early October, just after harvest in both places. Collections were made, without using any baits, by net sweeps over fallen apples or grape remains, which are natural substrates for the species just after harvest, when the *Drosophila* flies were collected. Females and males were immediately separated, and the females were individually placed in separate culture vials, where they were allowed to lay eggs for 5 days (Davis) or 7 days (Requena). These females and their F₁ progenies, as well as the wild collected males, were frozen at –80°C (Davis) or –30°C (Requena) until used for gel electrophoresis.

We used four polymorphic genes coding for enzymes as markers: alcohol dehydrogenase (*Adh*), α -glycerol-3-phosphate dehydrogenase (*Gpdh*), esterase-c (*Est-C*), and phosphoglucosyltransferase (*Pgm*). In *D. melanogaster*, *Adh* (II, 50.1) and *Gpdh* (II, 17.8) are on the second chromosome; *Est-C* (III, 47.7) and *Pgm* (III, 43.4) are on the third chromosome. We followed the methods of Ayala *et al.* (16) for starch gel electrophoresis and enzyme assay.

We used two different theoretical models for ascertaining CMP. The Milkman and Zeitler (MZ) method (17) requires that the genotype of the mother be known by direct ascertainment. The array of genotypes in the F₁ descendants is used to infer whether they were fathered by more than one male. The formula we have used in our calculations is slightly modified to give it greater generality than the one proposed by Milkman and Zeitler. They estimate CMP by calculating the probability that either three or four different male gametes are represented in the progeny; we calculate the probability that more than two such gametes are represented. With the MZ method, multiple paternity can be detected only when a gene marker is represented by more than two alleles. *Est-C* and *Pgm* meet this requirement in both populations, but not *Adh* or *Gpdh* (Table 1). A third *Gpdh* allele occurs with a frequency around 0.02 in the Davis population, much too low to be useful for the present purpose. However, given that no male recombination occurs in *D. melanogaster*, the allele frequencies of the two genes located

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CMP, concurrent multiple paternity; MZ, Milkman and Zeitler (ref. 17); WE, Williams and Evarts (ref. 18).

‡To whom reprint requests should be addressed.

Table 1. Genetic polymorphism at four loci in Davis, California, and Requena, Spain

Locus	N	Allele frequency								H-W	Heterozygote frequency		P
		1	2	3	4	5	6	7	8		Observed	Expected	
Davis, California													
<i>Adh</i>	419	0.667	0.333							<0.05	0.499	0.444	<0.05
<i>Gpdh</i>	289	0.822	0.159	0.019						<0.05	0.225	0.299	<0.05
<i>Est-C</i>	402	0.687	0.300	0.007	0.006					NS	0.411	0.439	NS
<i>Pgm</i>	417	0.835	0.106	0.040	0.010	0.006	0.002	0.001	0.001	NS	0.276	0.291	NS
Requena, Spain													
<i>Adh</i>	488	0.729	0.269	0.002						NS	0.400	0.397	NS
<i>Gpdh</i>	541	0.696	0.304							<0.05	0.335	0.423	<0.05
<i>Est-C</i>	532	0.768	0.161	0.032	0.039					<0.05	0.278	0.382	<0.05
<i>Pgm</i>	570	0.867	0.096	0.010	0.013	0.000	0.000	0.006	0.008	NS	0.242	0.239	NS

N is number of individuals assayed. Alleles are listed in order of decreasing frequency in the Davis population. H-W gives the χ^2 probability that the observed genotype frequencies are in Hardy-Weinberg equilibrium. P is the χ^2 probability for the agreement between the observed and expected (calculated according to Hardy-Weinberg equilibrium) frequency of heterozygotes. NS, not significant.

in each chromosome can be combined into gametic (haplotype) classes. There are four or more haplotype combinations at each locus, which can be used as so many "alleles" in estimating CMP by the MZ method. We have obtained four separate estimates of CMP by this method. Two estimates are derived from separate consideration of *Est-C* and *Pgm*. Two other estimates are derived from separate consideration of the two sets of haplotypes (*Adh* plus *Gpdh* and *Est-C* plus *Pgm*).

The incidence of CMP directly inferred from joint consideration of the genotypes of the mother and her progeny is an underestimate of the true CMP. Multiple paternity cannot be detected in the case of certain combinations of maternal and paternal genotypes (e.g., when two fathers have identical genotypes, or when each is homozygous for a different allele, etc.). Therefore, the "detected" frequency of CMP has to be corrected by dividing it by the probability that multiple paternity can be detected at all (which probability is calculated from the allele frequencies in the population; see ref. 17). We have used this correction only for the haplotype analysis. The two most common alleles at either *Est-C* and *Pgm* have a joint frequency >90%, which makes the probability of CMP detection based on each locus separately very low, and thus subject to very large statistical error, which would be magnified by the correction factor.

We have also ascertained CMP by the Williams and Evarts (WE) method (18). This method, like MZ, requires that the mother's genotype be known. The WE method is based on the distribution of genotypes among the progeny (given the genotype of the mother), rather than on the presence of extra progeny genotypes that would require insemination by more than one male, which is the case of MZ. The WE method is particularly efficient when the two most common alleles have a joint frequency >0.90, as is the case for the four markers in both populations. The efficiency decreases relative to MZ as more and more than two alleles are present at frequencies >0.05. We have used the WE method also with the haplotype combinations, but with little gain relative to the information obtained separately from each locus.

To test whether CMP is different between the two populations, we have used standard statistical methods, such as analysis of variance and Student's *t* test, calculated with the estimated frequencies, as well as arcsine-transformed frequencies. Since the statistical distribution of the CMP estimates is not known, we have also obtained bootstrap confidence intervals (19) based on 2000 repetitions. We have carried out the bootstrap resampling in two ways. First, we resampled the family data set—i.e., the set in which each entry consists of the genotype of the mother plus the genotypes of her progeny. Second, we resampled also the set of genotypes (usually 10) in each progeny array. We have performed the statistical tests

only on the WE estimates of CMP, since we judge that the MZ estimates are too skimpy and unreliable.

We have also tested whether the CMP estimates obtained for each population (or both) with different markers are statistically homogeneous—i.e., whether they may be considered to come from the same, randomly sampled normal distribution. We have used the Shapiro-Wilk (20) test for normality as implemented in the JMP statistical package, version 2.0.5 (SAS Institute, Cary NC).

RESULTS

Table 1 gives the allele frequencies at four polymorphic loci that serve as genetic markers in the two localities studied, Davis (California) and Requena (Spain). Several hundred wild-caught individuals have been assayed at each locus in each of the two populations. In all cases, we observe a predominant allele and a second common allele. Several rare alleles occur in *Est-C* and *Pgm*, with a collective frequency <0.10 at either locus. The genotype frequencies at two loci in each population depart significantly from the Hardy-Weinberg expectations (H-W column in Table 1). These deviations are largely due to differences in the frequency of heterozygotes, which are greater than expected at *Adh* in California, but lower than expected in the other three significant cases. We do not know the explanation for these discrepancies in heterozygote frequency, but they are of no consequence for estimating CMP by the MZ method, and they are likely to have little effect on the WE estimates, which use Hardy-Weinberg values derived from the allele frequencies.

The females we have used for ascertaining CMP were collected without bait in natural populations and immediately placed individually in culture vials. The genotype of the mother and 10 of her *F*₁ progeny (the progeny array) was ascertained at each of four loci, each of which provides an independent estimate of CMP. The estimates obtained by the MZ method (17) are given in Table 2. This method detects multiple paternity by noticing that more *F*₁ genotypes occur than can be accounted for by only one father, given the genotype of the mother. Whenever only two alleles occur at a locus, multiple paternity cannot be detected at all by this method. This situation obtains at the *Adh* and *Gpdh* loci in both populations. (The presence of a third allele at a frequency of about 0.02 at *Gpdh* in Davis is of little use.) Instances of CMP are detected in both populations with each of the other two markers, *Est-C* and *Pgm*, but they are very few. The probability of detecting CMP is indeed very low, because alleles other than the two most common ones occur at very low frequency.

The genotype at each of the four markers is known for every progeny array assayed from Requena and for a large sub-

Table 2. Frequency of CMP, estimated by the MZ method, in two natural populations of *D. melanogaster*

Locus	Davis, California				Requena, Spain			
	N	Detected frequency	Detection probability	Estimated frequency	N	Detected frequency	Detection probability	Estimated frequency
<i>Adh</i>	419	0	0	—	66	0	0	—
<i>Gpdh</i>	289	0	0	—	66	0	0	—
<i>Est-C</i>	402	0.007 ± 0.004			62	0.113 ± 0.040		
<i>Pgm</i>	417	0.010 ± 0.005			62	0.003 ± 0.022		
<i>Adh</i> and <i>Gpdh</i>	92	0.174 ± 0.040	0.374	0.464 ± 0.052	66	0.167 ± 0.046	0.462	0.361 ± 0.059
<i>Est-C</i> and <i>Pgm</i>	125	0.112 ± 0.028	0.378	0.296 ± 0.041	62	0.290 ± 0.058	0.325	0.894 ± 0.039

N is the number of wild females tested. Detection probability is the probability that remating will be detected when it has occurred. The standard errors are based on Milkman and Zeitler (17).

sample from Davis. In these cases, we can examine simultaneously the two markers from each chromosome, which considerably increases the number of “alleles” (haplotypes) that occur at frequencies >0.05. The cases of CMP detected in this manner are reported in the two bottom rows of Table 2. The probability of detecting CMP whenever it has occurred is, even in this case, fairly low, 0.325–0.462, so that the estimates of CMP may be subject to considerable statistical error. This is particularly apparent in the Requena population, in which the females tested by the two sets of markers are virtually identical (four additional females are tested with the chromosome II markers). The two sets of markers are estimating the same population parameter, yet the chromosome II markers indicate that 0.639 of the females had progeny fathered by only one male (CMP = 0.361), whereas the chromosome III markers indicate only 0.106 (CMP = 0.894), one sixth as many. The CMP estimates obtained with the two sets of markers are also quite different in the Davis population (0.296 and 0.464).

The WE (18) method estimates CMP by inference from the distribution of genotypes in the progeny array, given the genotype of the mother and the allele frequencies in the population. This method relies in effect on only two allele frequency values, the frequency of the most common allele and the combined frequency of all other alleles, and is most informative when these two values are or approach 0.50. Compared with the MZ method, the WE method is more effective when only two alleles are present in the population, or when the two most common alleles have a combined frequency >0.90, as is the case for each of the four genetic markers in both populations. Only 100 females and progeny arrays were analyzed by the WE method in the Davis population, rather than 289–419 by the MZ method; 62–66 were analyzed in the Requena population, the same as with the MZ method. The CMP estimates obtained by the WE method are given in Table 3. The estimates obtained with each of the four genetic markers are fairly homogeneous, in either population,

with a mean 0.555 ± 0.071 in Davis and 0.521 ± 0.056 in Requena. The relative homogeneity of the values obtained with the different markers is reassuring, since each marker estimates the same population parameter. A Shapiro–Wilk (20) test indicates that the set of CMP estimates obtained with the four markers is not statistically heterogeneous, in Davis as well as in Requena. Combining the allele frequencies for the two genes in each chromosome yields little improvement with the WE method, due to the fact that the combined haplotype frequencies are only slightly closer to 0.50 than the separate allele frequencies. The mean CMPs for the haplotype data are 0.566 ± 0.081 and 0.501 ± 0.062 for Davis and Requena, respectively. A Shapiro–Wilk (20) test indicates that the four single-marker estimates plus the two combined estimates can statistically be accepted as randomly sampled from the same normal distribution, with mean and standard deviation of 0.558 ± 0.121 (Davis), 0.514 ± 0.095 (Requena), and 0.536 ± 0.107 (both populations combined).

We have tested in a variety of ways whether the CMP estimates for Davis and Requena are statistically different. All tests consistently fail to demonstrate significant differences between the two populations in the incidence of CMP. The tests include analyses of variance among the four single-locus estimates ($F = 0.14$, $P > 0.72$; homoscedasticity not significant) and their arcsine-transformed values ($F = 0.16$, $P > 0.71$; homoscedasticity not significant); between the two chromosome haplotype estimates ($F = 0.41$, $P > 0.59$) and their arcsine-transformed values ($F = 0.42$, $P > 0.58$); among the set of six estimates made up of the four single-locus plus the two haplotype values ($F = 0.49$, $P > 0.50$; homoscedasticity not significant) and their arcsine-transformed values ($F = 0.52$, $P > 0.49$; homoscedasticity not significant). Similarly, paired *t* tests all indicate that differences between the two populations are not significant: between the four single locus pairs and their arcsine-transformants ($t = 0.56$ and 0.63 , respectively); between the two haplotype pairs and their transformants ($t =$

Table 3. Frequency of CMP, estimated by the WE method, in two natural populations of *D. melanogaster*

Locus	Davis, California			Requena, Spain		
	N	CMP frequency	95% C.I.	N	CMP frequency	95% C.I.
<i>Adh</i>	100	0.535 ± 0.014	0.251–0.804	66	0.404 ± 0.037	0.147–0.592
<i>Gpdh</i>	100	0.389 ± 0.014	0.168–0.736	66	0.532 ± 0.021	0.263–0.746
<i>Est-C</i>	100	0.735 ± 0.013	0.422–0.932	62	0.668 ± 0.018	0.345–0.946
<i>Pgm</i>	100	0.559 ± 0.016	0.269–0.875	62	0.479 ± 0.018	0.126–0.751
Mean		0.555 ± 0.071			0.521 ± 0.056	
<i>Adh</i> and <i>Gpdh</i>	92	0.485 ± 0.007		66	0.439 ± 0.006	
<i>Est-C</i> and <i>Pgm</i>	100	0.647 ± 0.007		62	0.563 ± 0.010	
Mean		0.566 ± 0.081			0.501 ± 0.062	

N is the number of wild females tested. CMP frequencies are given ± the standard errors based on Williams and Evarts (18), except for the means, which are obtained from the variance among estimates. The 95% C.I. are confidence intervals obtained by bootstrap resampling with 2000 pseudoreplications. The 95% C.I. can be corrected for bias by the method of Efron and Tibshirani (ref. 19, pp. 124–139), but this correction affects only the second decimal digit of the C.I. and does not change the absence of statistical significance for the comparisons between populations or loci.

3.42 and 2.93, $P \approx 0.09$ and 0.11, respectively); and between the combined set of six values and their arcsine-transformants ($t = 1.13$ and 1.23).

All the previous tests are performed on the sample estimates, ignoring the standard errors calculated by the WE method (18), since the distribution of the WE statistic is unknown. But we have also performed bootstrap resampling of each set of observations. Table 3 gives the 95% confidence intervals obtained with 2000 resamplings of the data for each of the four loci in each population. There are not significant differences between Davis and Requena (nor are the four estimates from each population significantly heterogeneous).

As can be seen in Table 3, the 95% confidence intervals obtained by bootstrap resampling are much wider than those that would be obtained by doubling the standard errors calculated by the WE method. These standard errors would, indeed, be inappropriate for statistical tests of significance either between markers or between populations.

DISCUSSION

We have shown that *D. melanogaster* females are often inseminated by more than one male, retain their sperm, and concurrently use it to produce progeny fathered by more than one male. We have estimated that CMP occurs in somewhat more than half the progeny arrays tested: 0.56 ± 0.07 in an apple orchard near Davis, California, and 0.52 ± 0.06 in a winegrape vineyard from Requena, Spain (Table 3). These are likely to be underestimates of the incidence of the phenomenon in nature for two reasons. One reason is that we have kept the females individually isolated for 5–7 days while they laid eggs, unable to establish contact and perhaps mate with additional males. A second reason is that we have assayed from each progeny array only 10 individuals, among the hundreds produced by each female. Assaying additional F_1 individuals would likely have identified additional cases of CMP. (This bias, however, has been corrected in part for the WE estimates by the bootstrap calculations. The “probability of detection” used with the MZ method, however, is a correction parameter that refers to the likelihood of genetic ascertaining more than one father, given that 10 F_1 progeny have been observed; it is not a correction factor for the number of progeny examined.) The inference of 52–56% of CMP cases may thus be seen as the lower-bound of the incidence of CMP in nature.

Nonidez (21) discovered more than seven decades ago that *D. melanogaster* females may carry sperm from more than one male. He also discovered the phenomenon now known as sperm displacement—i.e., that the sperm of the second mating male tends to be used first and that the sperm of the first male may be used again when that of the second male approaches depletion. Lefevre and Jonsson (22) directly observed sperm displacement in the female reproductive organs of *D. melanogaster*. Dobzhansky and Pavlovsky (23) observed that about 50% of *Drosophila pseudoobscura* females produced progeny fathered by more than one male, but their experiments were performed in rather crowded and contrived circumstances in the laboratory. CMP in laboratory populations has also been observed in other *Drosophila* species, such as *D. melanogaster* (24) and *D. paulistorum* (25).

Anderson (26) studied CMP in wild-collected *D. pseudoobscura* females, and estimated that about half produced progeny fathered by more than one male. Milkman and Zeitler (17) extended to *D. melanogaster* the observation of CMP in wild-collected females, with an incidence approaching 50%. Cobbs (14) observed different CMP frequencies, ranging from 0.429 to 0.698 in three samples of *D. pseudoobscura* females collected in the same locality at different times over a period of 2½ years; but the standard errors of the estimates are much too large to warrant the inference that CMP was different at different times.

The occurrence of CMP in natural or laboratory populations has now been extended to diverse *Drosophila* species, such as *D. subobscura* (27), *D. affinis*, and *D. athabasca* (28), which like *D. melanogaster* and *D. pseudoobscura*, belong to the *Sophophora* subgenus, as well as to *D. euronotus* (29), *D. silvestris* (30), *D. buzzatii* (31), and *D. virilis* (32), which belong to the *Drosophila* subgenus.

The incidence of CMP in wild-caught females is often around 0.50, as in the examples cited. Levine *et al.* (15), however, estimate the incidence of CMP in a Mexican population of *D. pseudoobscura* to be around 0.90 with 95% confidence interval of 0.62–1.00. They separated females from males after collection, but the flies were collected over fermenting banana baits, where flies aggregate, which may have artificially increased the incidence of multiple matings. Marks *et al.* (13) have, nevertheless, demonstrated that the incidence of CMP may vary as a function of habitat. They collected *D. melanogaster* flies, shortly after harvest, by net sweeping inside a California winery building, and in fermenting banana traps set among oak trees outside the winery. The CMP incidence was significantly higher inside than outside the winery. This may have been due to the much higher density of flies inside than outside. But Gromko and Gerhart (33) and Harshman *et al.* (34) have failed to demonstrate a positive association between density and CMP frequency. In our experiment, the two habitats are very different, as evidenced, e.g., by species composition: in the apple orchard, more than 60% of all *Drosophila* flies collected are *D. simulans*, with *D. melanogaster* representing only a modest fraction of the rest; in the vineyard, 88% are *D. melanogaster* and 11% *D. simulans*. Moreover, large density differences occur between the apple orchard in Davis and the vineyard in Requena, where collections were made in the proximity of the winery building, just after harvest when the density of *D. melanogaster* flies is surely orders of magnitude higher than in the Davis orchard. Yet we have failed to demonstrate significant differences between the two populations in the CMP frequency.

CMP and sperm displacement have now been observed in numerous insect species, such as the Drosophilid *Chymomyza amoena* (35), the dung fly *Scatophaga stercoraria* (36), several species of fleas (37), and the beetle *Aleochara curtula* (38), as well as in the millipede *Orthoporus pyrhocephalus* (39). Ridley (40) has listed 57 insect species for which there is evidence of sperm displacement. He has investigated four not mutually exclusive hypotheses to explain why the degree of sperm displacement is greater in some than in other species, and concluded that the evidence favors the “mating frequency” hypothesis: the greater the female’s frequency of mating in a species, the larger the degree of sperm displacement.

The male–female adaptive interactions associated with mating are very complex (41, 42). In *Drosophila*, effects of the seminal fluid include inhibiting other males’ sperm from fertilizing the eggs (5, 6) and reducing the female’s propensity to remate (7), with a consequent increase in male fitness. But the seminal fluid has substantial toxic effects in females, so that greater amounts of fluid increasingly reduce female survival (8, 9, 43). The longevity of females is significantly lower in those that mate two or more times than in those that mate only once (3).

Rice (4) has dramatically demonstrated the tension between the conflicting interests of males and females. When *D. melanogaster* females are experimentally prevented from evolving, males rapidly evolve an increased rate of remating and greater toxicity of their seminal fluid, attributes that positively contribute to male fitness but hamper the fitness of the females (7, 10, 42). Traditionally, evolutionists have been interested in female fecundity as an important component of fitness. This is, of course, correct, but it is becoming increasingly apparent that the contributions of male mating success to

fitness are complex and important; in some cases they are of greater evolutionary consequence than female fecundity (2).

We are grateful to Laurence D. Mueller, Steve Rich, Garr Updegraff, and Pedro Cuesta for statistical advice and computational help, and to Wyatt W. Anderson, Laurence D. Mueller, and Antonio Prevosti for valuable comments on the manuscript. This work has been supported by National Institutes of Health Grant GM42397 and by the Fundación Del Amo-Universidad Complutense de Madrid.

1. Bodmer, W. F. (1965) *Genetics* **51**, 411–424.
2. Brockett, M. M., Alavi, H. & Anderson, W. W. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3080–3082.
3. Turner, M. E. & Anderson, W. W. (1983) *Evolution* **37**, 714–723.
4. Rice, W. R. (1996) *Nature (London)* **381**, 232–234.
5. Clark, A. G., Aguade, M., Prout, T., Harshman, L. G. & Langley, C. H. (1995) *Genetics* **139**, 189–201.
6. Harshman, L. G. & Prout, T. (1994) *Evolution* **48**, 758–766.
7. Chen, P. S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M. & Bohlen, P. (1988) *Cell* **54**, 291–298.
8. Trevitt, S. & Partridge, L. (1991) *J. Insect Physiol.* **37**, 471–475.
9. Chapman, T., Liddle, L. F., Kalb, M. J., Wolfner, M. F. & Partridge, L. (1995) *Nature (London)* **373**, 241–244.
10. Markow, T. A. (1996) *Evol. Biol.* **29**, 73–106.
11. Scott, D. & Richmond, R. C. (1990) *J. Insect Physiol.* **36**, 451–456.
12. Band, H. T. (1996) *Evol. Biol.* **29**, 151–214.
13. Marks, R. W., Seager, R. D. & Barr, L. G. (1988) *Am. Nat.* **131**, 918–923.
14. Cobbs, G. (1977) *Am. Nat.* **111**, 641–656.
15. Levine, L., Asmussen, M., Olvera, O., Powell, J. R., de la Rosa, M. E., Salceda, V. M., Gaso, M. I., Guzman, J. & Anderson, W. W. (1980) *Am. Nat.* **116**, 493–503.
16. Ayala, F. J., Powell, J. R., Tracey, M. L., Mourão, C. A. & Pérez-Salas, S. (1972) *Genetics* **70**, 113–139.
17. Milkman, R. & Zeitler, R. R. (1974) *Genetics* **78**, 1191–1193.
18. Williams, C. J. & Everts, S. (1989) *Theor. Popul. Biol.* **35**, 90–112.
19. Efron, B. & Tibshirani, R. J. (1993) *An Introduction to the Bootstrap* (Chapman & Hall, New York).
20. Shapiro, S. S. & Wilk, M. B. (1965) *Biometrika* **52**, 591–611.
21. Nonidez, J. F. (1920) *Biol. Bull.* **39**, 207–230.
22. Lefevre, G. & Jonsson, U. B. (1962) *Genetics* **47**, 1719–1736.
23. Dobzhansky, Th. & Pavlovsky, O. (1967) *Am. Nat.* **101**, 527–533.
24. Fuerst, P. A., Pendlebury, W. W. & Kidwell, J. F. (1973) *Evolution* **27**, 265–268.
25. Richmond, R. C. & Ehrman, L. (1974) *Experientia (Basel)* **30**, 489–490.
26. Anderson, W. W. (1974) *Am. Nat.* **108**, 709–711.
27. Loukas, M., Vergini, Y. & Krimbas, C. B. (1981) *Genetica* **57**, 29–37.
28. Gromko, M. H., Sheehan, K. & Richmond, R. C. (1980) *Am. Nat.* **115**, 467–479.
29. Stalker, H. T. (1976) *Genetics* **84**, 375–384.
30. Craddock, E. M. & Johnson, W. E. (1978) *Drosophila Inf. Serv.* **53**, 138–139.
31. Barbadilla, A., Quezada-Díaz, J. E., Ruiz, A., Santos, M. & Fontdevila, A. (1991) *Genet. Sel. Evol.* **23**, 133–140.
32. Aspi, J. (1992) *Ann. Zool. Fenn.* **29**, 147–159.
33. Gromko, M. H. & Gerhart, P. D. (1984) *Evolution* **38**, 451–455.
34. Harshman, L. G., Hoffmann, A. A. & Prout, T. (1988) *Evolution* **42**, 312–321.
35. Band, H. T. (1995) *Bull. Soc. Entomol. Suisse* **68**, 23–33.
36. Parker, G. A. & Simmons, L. W. (1991) *Proc. R. Soc. London Ser. B* 107–115.
37. Rothschild, M. L. (1991) *Biol. J. Linn. Soc.* **43**, 313–323.
38. Gack, C. & Peschke, K. (1994) *Zoomorphology* **114**, 227–237.
39. Barnett, M., Telford, S. R. & de Villiers, C. J. (1993) *J. Zool. (London)* **231**, 511–522.
40. Ridley, M. (1989) *Biol. J. Linn. Soc.* **38**, 349–367.
41. Gromko, M. H., Gilbert, D. G. & Richmond, R. C. (1984) in *Sperm Competition and the Evolution of Animal Mating Systems*, ed. Smith, R. L. (Academic, New York), pp. 371–426.
42. Pitnick, S. & Markow, T. A. (1994) *Am. Nat.* **143**, 785–819.
43. Fowler, K. & Partridge, L. (1989) *Nature (London)* **338**, 760–761.