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An Experimental Test for Synergistic Epistasis and Its Application in Chlamydomonas

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ABSTRACT

Theoretically, one of the most general benefits of sex is given by its function in facilitating selection against deleterious mutations. This advantage of sex may be deterministic if deleterious mutations affect the fitness of an individual in a synergistic way, *i.e.*, if mutations increase each others' negative fitness effect. We present a new test for synergistic epistasis that considers the skewness of the log fitness distribution of offspring from a cross. We applied this test to data of the unicellular alga *Chlamydomonas moewussii*. For this purpose, two crosses were made: one between two strains that are presumed to have accumulated slightly deleterious mutations, the other between two strains without a history of mutation accumulation. Fitness was measured by estimating the two parameters of logistic growth in batch culture, the maximum growth rate (r) and the carrying capacity (K). The finding of a negatively skewed distribution for K in the accumulation cross suggests synergism between mutations affecting the carrying capacity, while the absence of skewness for r in both crosses is consistent with independent effects of mutations affecting this parameter. The results suggest a possible alternative explanation for the general observation that sex is related to constant environments, where selection on K predominates, while asexual reproduction is found in more variable environments, where selection on r is more important.

TO explain the ubiquity of sexual reproduction, a short-term advantage of sex is needed that may compensate its twofold disadvantage (MAYNARD SMITH 1971). One of the most attractive advantages of sex is provided by the Deterministic Mutation Hypothesis (CROW 1970; KONDRASHOV 1988). Its attraction is due to its general validity, but also to its clear-cut assumptions: the rate at which deleterious mutations occur should be at least one per genome per generation (KONDRASHOV 1982; CHARLESWORTH 1990) and deleterious mutations should have synergistic effects on fitness, *i.e.*, they should amplify each others' negative effects (KIMURA and MARUYAMA 1966; CROW 1970; KONDRASHOV 1982; CHARLESWORTH 1990). At present, data both on mutation rate and on epistasis between deleterious mutations are too scarce to be conclusive (KONDRASHOV 1993).

Here, we present a new test for epistasis between deleterious mutations. Due to random segregation and recombination, a sexual cross between two individuals (who carry an unknown number of deleterious mutations) produces a symmetrical distribution of mutation number among the offspring, with the mean equal to that of the parents. In a population with a symmetrical distribution of mutation number, skewness of the fitness distribution provides information on mutation interaction: negative skewness reflects synergism, positive skewness reflects antagonism, *i.e.*, the situation where mutations decrease each others' negative fitness effects.

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The distribution of the *logarithm* of fitness should be considered, because mutations with multiplicative effects, *i.e.*, no interaction (KONDRASHOV 1988; CHARLESWORTH 1990), will cause the distribution of log offspring fitness to be symmetrical. Deviation from symmetry, *i.e.*, skewness, can then be used as indicative of nonmultiplicative mutation effects. In Figure 1, the test is illustrated for the simplistic situation of all mutations having equal effect. However, since half of the offspring receive each mutation carried by a parent and the other half does not receive this mutation, nonequal mutation effects will also lead to no skewness if mutations show no interactions.

The relation between synergism and negative skewness can be derived analytically, as follows. Let x be a symmetrically distributed trait (in our case the number of mutations carried by an offspring) and let y be a function of x, $g(x)$ (in our case log fitness). Deviations of x and y from their mean are represented by δx and δy. Then, δy can be approximated by the first two terms of the Taylor expansion:

$$\delta y \approx (\delta x)g' + 0.5(\delta x)^2 g'' \qquad (1)$$

primes denote derivatives evaluated at the mean. The skewness of y can then be expressed as the expectation of $(\delta y)^3$ in which the odd moments of x are dropped:

$$E\{(\delta y)^3\} \approx 1.5(g')^2(g'')E\{(\delta x)^4\} + 0.125(g'')^3 E\{(\delta x)^6\} \qquad (2)$$

which is <0 if $g'' < 0$. The latter condition corresponds to a concave relationship between log fitness and mutation number, *i.e.*, synergism between deleterious mutations.

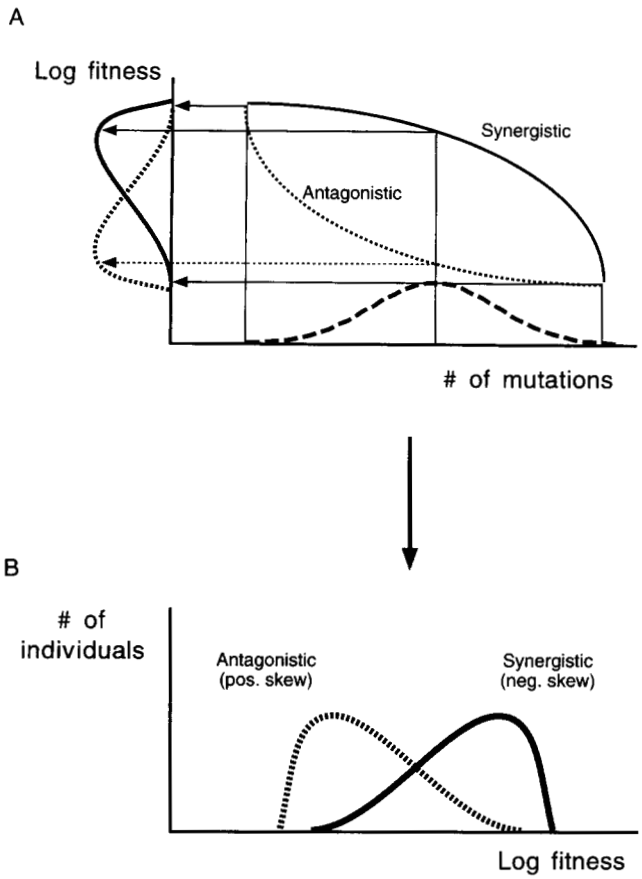

FIGURE 1. The skewness test for epistasis between deleterious mutations, illustrated with graphical arguments for the situation of mutations having all equal effects. (A) Mapping the symmetrical mutation distribution among sexual offspring of two parents (dashed curve on *x*-axis) on a concave curve relating mutation number and log fitness, which represents synergism between mutations, results in a negatively skewed log fitness distribution (solid distribution on *y*-axis). Mapping this distribution on a convex curve, representing antagonistic mutation interaction, yields a positively skewed log fitness distribution (dotted distribution on *y*-axis). (B) A negatively skewed log fitness distribution (——) among full-sib offspring is indicative of synergistic mutation interaction, a positively skewed distribution (· · ·) reflects antagonistic mutation interaction.

We applied the skewness test to data of two crosses between strains of the haploid unicellular alga *Chlamydomonas moewusii*. One cross, the "accumulation cross", was made between strains that have been kept separate in the laboratory for >60 yr. Since the species is heterothallic, no sex occurred during this period. The frequent transfer (every 3–6 mon) of a small sample (containing on the order of 10^4 viable cells) of each strain to fresh medium makes it likely that deleterious mutations will have accumulated due to Muller's ratchet (MULLER 1964; HAIGH 1978). The size of the least mutated class of cells in this sample is given by $n_0 = Ne^{(U/s)}$ (HAIGH 1978), where N = bottle-neck population size, U = the genomic deleterious mutation rate, and s = the mean selection coefficient of mutations. If $n_0 < 1$, this class will probably be lost during each transfer. Using conservative estimates for U ("per transfer": 0.2) and s (0.02), n_0 in our strains is 0.5, making the frequent loss of this class likely. Moreover, additional mutations with very small deleterious effects may have become fixed by genetic drift, since they are effectively neutral, given the relatively small population size of the strains (KIMURA 1983). Another cross, the "control cross", was made between two strains that were recently isolated from nature (in 1990) and, therefore, had not had the opportunity to accumulate many mutations. The fitnesses of individual offspring of both crosses were measured by estimating the two parameters of the logistic growth model, maximum growth rate (r) and carrying capacity (K) in batch culture (*cf.* BELL 1990).

The results suggest synergism between deleterious mutations that affect K, while the absence of skewness for r is most consistent with multiplicative effects between mutations that affect this parameter. If the differing results for r and K hold more generally, they support an alternative explanation for the ecological distribution of sex by the Deterministic Mutation Hypothesis.

MATERIALS AND METHODS

Strains: The following strains of *C. moewusii* were used: strains UTEX 9 (mating-type plus: mt^+) and UTEX 10 (mating-type minus: mt^-) (HARRIS 1989), which were isolated around 1930 (E. H. HARRIS, personal communication), in the accumulation cross and the recently isolated strains SAG 23.91 (mt^+) and SAG 24.91 (mt^-) (SCHLOESSER 1994) in the control cross. Each of the strains originated from a single cell at the time of isolation and was ever after maintained clonally by growing them on agar slants containing 3 ml of solid M1 minimal salts medium (MESLAND 1976). Every 3–6 mo a sample containing $\sim 10^4$ viable cells was transferred to fresh medium. These stock cultures were allowed to grow for 1 mon, after which time the passive diffusion of air was stopped by firmly closing the screw-caps of the tubes. The maximum cell number reached in the agar slants was estimated to be on the order of 10^7.

Crossing protocol: We used the protocol of SCHURING *et al.* (1987) to isolate sexual offspring from both crosses. For the production of gametes, typically on the order of 10^4 viable cells are taken from the stock-strain and grown on solid medium, resulting into 10^6–10^7 gametes, *i.e.*, some 10 cell generations, after flooding the culture with water. Mutations that occur during gamete production may contribute to the mutation variation among the offspring. However, their contribution should be negligible relative to the mutations that accumulated during the thousands of generations while the strains were maintained in the lab. Selection will further reduce the probability that zygotes were isolated that were produced by mutant gametes. Mutation variation among the gametes of one strain can, therefore, be neglected as a source for genetic fitness differences among the offspring, compared with the mutation variation between the two parental strains. Crossing the two recently isolated strains of *C. moewusii* (SAG 23.91 and SAG 24.91) resulted in more offspring than crossing the two old strains (UTEX 9 and UTEX 10): 176 *vs.* 72 offspring. The offspring of the control cross were all isolated in one crossing experiment, while the offspring of the accumulation cross were the total of three independent attempts to isolate

offspring from these old strains. After isolation, offspring were kept on agar slants with 3 ml solid M1 medium.

Measuring fitness: We used the two parameters for logistic growth, the maximum growth rate (r) and the carrying capacity (K), of each offspring as measure of fitness. The idea of measuring r and K in a test tube is derived from BELL (1990). However, the logistic growth model is not an appropriate model for a closed system like a batch culture and, therefore, our estimate of K should rather be interpreted as the total yield from a given amount of nutrients (R. E. LENSKI, personal communication).

All offspring and parents were transferred to a multiwell plate with M1 liquid medium for 4 days, and the cell densities of these cultures were equalized by measuring absorbance at 405 nm on a plate reader. Then 15-μl aliquots, containing ~600 cells, were taken to inoculate two test tubes of 6 ml M1 liquid medium for each offspring, and ten test tubes for each parent. All 536 test tubes (72 offspring of the accumulation cross and 176 offspring of the control cross, all replicated twice, and four parents replicated in 10-fold) were randomized over 12 racks and placed under continuous light (fluence rate 40 $J \cdot m^{-2} \cdot s^{-1}$) at 21° for 3 mon. Test tubes were briefly vortexed daily (after 6 wk: three times a week) and absorbance at 660 nm was scored with a colorimeter (Corning colorimeter, model 257). Cell density (cells/ml) was inferred from the absorbance data in a dilution series by linear regression. Due to irregular growth of some of the cultures, maximum cell density was used as the estimate of K, while r was estimated from the data that were truncated before the first irregularities occurred. For the estimation of r we used the NONLIN procedure of SYSTAT (WILKINSON 1988) to fit the logistic growth model to the cell density data. Initial cell number was estimated as well. This improved the fit of the model, giving a higher explained variance after correction for estimating this additional parameter from the data.

Statistical analysis: The growth of 18 cultures of the control cross and 11 cultures of the accumulation cross had not stopped at the end of the growth period so that no accurate estimates of K for these cultures were available. Furthermore, for five cultures of the control cross and five cultures of the accumulation cross, no valid estimate of r could be generated, due to no or bad (corrected $r^2 < 0.8$) fit of the logistic growth model. This resulted in at least one estimate per genotype of K for 173 offspring in the control cross and for 71 offspring in the accumulation cross. At least one estimate of r per genotype was obtained for all 176 offspring in the control cross and for 71 offspring in the accumulation cross.

Analyses of variance were performed to estimate and test the significance of the genetic variance for fitness, V_G. For this purpose, only genotypes with two estimates of r or K were considered. For calculating the skewness of the log fitness distributions, first the arithmetic mean value of the two replicates was calculated (in case of one available replicate, this one value was used) and then the logarithm was taken. Next, the skewness of the log mean values was computed. To test the genetic contribution to the observed skewness, the significance of the skewness statistic g_1 of the log fitness distribution in the accumulation cross is tested relative to that in the control cross. For this purpose a two-tailed t-test was performed, assuming a normal distribution of the skewness difference and independence of the fitness estimates in the two crosses: $t_s = (g_1, \text{accumulation} - g_1, \text{control})/(SE^2_{\text{accumulation}} + SE^2_{\text{control}})^{0.5}$, d.f. $= n_{\text{accumulation}} + n_{\text{control}} - 2$. The exact formula for the standard error of the skewness statistic g_1 was used (SOKAL and ROHLF 1981, p 139). Since we only had two replicates per genotype, we could not accurately test for homogeneity of variances. However, a significantly positive correlation exists between mean and variance per genotype for r (control cross: $\rho = +0.61$, $n = 175$, $P < 0.001$; accumulation cross: $\rho = +0.38$, $n = 68$, $P < 0.01$). For K, the correlation between mean and variance is only slightly positive (control cross: $\rho = +0.15$, $n = 173$, $P < 0.05$; accumulation cross: $\rho = +0.16$, $n = 68$, $P > 0.05$). The correlation between mean r and K values in both crosses appeared to be slightly negative (control cross: $\rho = -0.18$, $n = 173$, $P = 0.016$; accumulation cross: $\rho = -0.24$, $n = 70$, $P = 0.047$), indicating that r and K may not be fully independent measures of fitness.

RESULTS

Accumulation of deleterious mutations in control and accumulation cross: If the parents of the accumulation cross carry a higher number of deleterious mutations than the parents of the control cross, we expect a higher genetic variance for fitness in the accumulation cross. As expected, among the offspring of the accumulation cross the genetic variance for K is almost 14 times higher than among the offspring of the control cross (Table 1). The genetic variance for r is not significant in the control cross ($V_G = -5.40 \times 10^{-6}$, 95% confidence limits are -2.75×10^{-5} and 1.54×10^{-5}), due to a high error variance, but is significant in the accumulation cross. These results suggest that the higher genetic variance for fitness in the accumulation cross is due to the redistribution of accumulated deleterious mutations.

Epistasis between deleterious mutations: If deleterious mutations show interaction with respect to the fitness parameters, we expect the effect to be clear only in the accumulation cross, due to the large genetic variation in fitness in this cross relative to the control cross. In the control cross, the distribution of log K appears to be unskewed (Figure 2: $t_s = +0.33$, d.f. $= 172$, $P = 0.74$), while it is significantly negatively skewed in the accumulation cross ($t_s = -2.11$, d.f. $= 70$, $P = 0.038$), resulting in a nearly significant relative negative skewness of log K in the accumulation cross ($t_s = -1.95$, d.f. $= 245$, $P = 0.052$). These results suggest synergism between deleterious mutations that affect the carrying capacity. The distribution of log r appears to be unskewed, both in the control cross ($t_s = +0.55$, d.f. $= 175$, $P = 0.58$) and in the accumulation cross ($t_s = -1.04$, d.f. $= 70$, $P = 0.30$), making the difference in skewness in these two crosses non-significant (ts = -1.18, d.f. $= 245$, p $= 0.24$). These results suggest independent effects of deleterious mutations affecting the maximum growth rate.

DISCUSSION

The aim of this paper is twofold. We present a new test for the mode of interaction between deleterious mutations, and we present and discuss the results of a first application of this test to data of the unicellular alga *Chlamydomonas moewusii.*

The skewness test for mutation interaction: The skewness test for epistasis has a simple rationale and can be applied very generally. It does not rely on the additional assumption of a rank-order relationship be-

TABLE 1

Genetic variation in fitness (K and r) in the control and accumulation cross

Cross	Mean[a]	Genotypes MS	Genotypes d.f.	Error MS	Error d.f.	V_G	P
K							
Control	$1.51 \times 10^6 \pm 1.56 \times 10^4$	7.62×10^{10}	160	4.69×10^{11}	161	1.46×10^{10}	1.1×10^{-3}
Accumulation	$1.66 \times 10^6 \pm 5.70 \times 10^4$	4.84×10^{11}	61	7.65×10^{10}	62	2.04×10^{11}	5.5×10^{-12}
r							
Control	$2.53 \times 10^{-3} \pm 5.80 \times 10^{-4}$	1.41×10^{-4}	172	1.52×10^{-4}	173	(0)	0.69
Accumulation	$1.45 \times 10^{-3} \pm 6.60 \times 10^{-4}$	6.17×10^{-5}	67	2.47×10^{-5}	68	1.85×10^{-5}	1.1×10^{-4}

[a] Means ± SE.

tween mutation number and fitness used in an earlier test for epistasis by us (DE VISSER *et al.* 1996). The application of the skewness test is limited by only three conditions. First, it can be applied only to haploid sexual species with large progeny numbers per cross. In diploids, dominance may also affect the skewness. The relative contributions of dominance and nonallelic interaction effects on the overall skewness needs to be studied before the skewness test can be applied to diploid species. Second, the parents of a cross need to carry sufficient deleterious mutations to warrant a significant genetic contribution to the fitness variation among their offspring. Third, the offspring isolated from a cross should be a random sample including (a representative fraction of) offspring with many mutations. Missing individuals with many mutations, for instance because they are small or inviable, generates a positive skewness of the log fitness distribution. The third condition implies that finding a negatively skewed log fitness distribution will be conservative evidence for synergism, while finding positive skewness may be ambiguous.

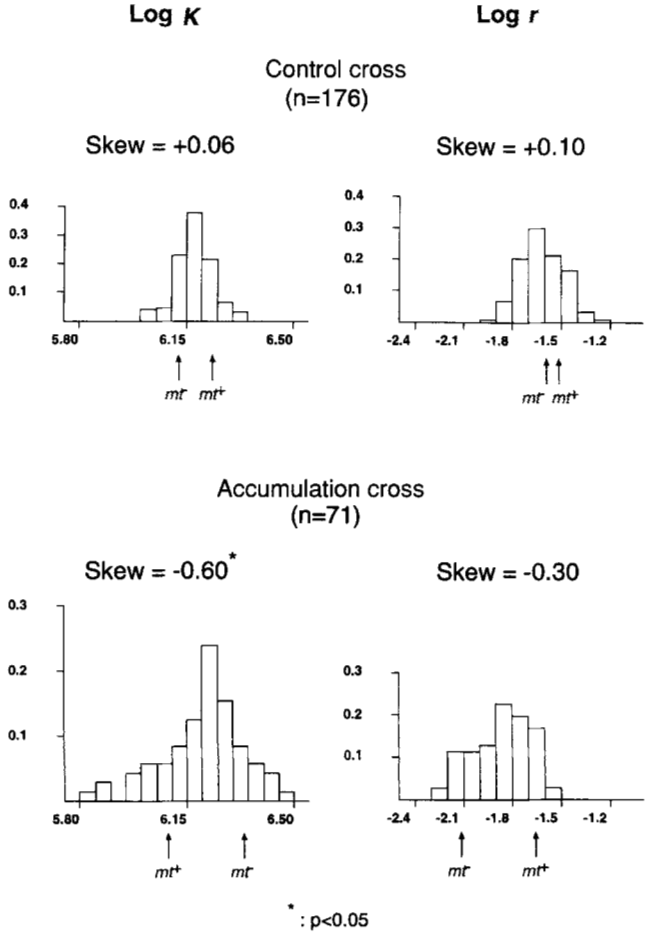

FIGURE 2. Log fitness distributions of control and accumulation cross. Individual estimates of log r and log K are the mean of two independent estimates of two replicate cultures. Mean log performance of the parental strains (mean of 10 replicates) and their mating type are indicated by arrows.

Application to data from Chlamydomonas: The results of the application of our test to data of *C. moewusii* are consistent with synergistic effects of deleterious mutations on the carrying capacity and independent effects on the maximum growth rate. A skewed distribution of the error may also have contributed to the negative skewness observed for K. However, the positive correlation found between mean and variance of the replicates for both r and K (see MATERIALS AND METHODS) suggests that the error distributions are positively rather than negatively skewed and, therefore, cannot explain the negative skewness observed for K. The results for K may, however, be stronger than the results for r for three reasons. In the first place, the genetic variance for K in the accumulation cross has a higher significance than the genetic variance for r, which emphasizes the stronger dependence of (the form of) the distribution of K on genetic differences between offspring. In the second place, a negatively skewed log fitness distribution will generally be conservative evidence for synergism, as explained above. In the third place, the rather low statistical power to find significant skewness makes conclusions drawn from the absence of skewness tentative.

How do the two parameters we estimated, r and K, relate to fitness? The maximum growth rate (r) of a specific offspring genotype estimated in monoculture can be compared with its Malthusian parameter when nutrients are available ad libitum. Under such conditions, growth limitation is mainly density independent and, therefore, maximum growth rate is comparable with relative fitness. The carrying capacity (K), as stated in MATERIALS AND METHODS, basically cannot be mea-

sured in batch culture and should rather be interpreted as the total yield of a specific offspring genotype. Moreover, since at carrying capacity density is high, growth limitation is mainly density dependent and K, estimated in monoculture, is not necessarily comparable to relative fitness in a competitive situation. HUISMAN and WEISSING (1994, 1995) and WEISSING and HUISMAN (1994), however, argue on theoretical grounds, that when light is the limiting nutrient, the relative fitness of an algal monoculture is linearly proportional to the fraction of light that is absorbed by the culture. Since our estimate of K is based on the maximum absorbance (at 660 nm) of a monoculture, K is at least comparable with relative fitness under high density conditions when light is limiting growth.

In an earlier paper (DE VISSER *et al.* 1996), where we used a test for epistasis that considered the difference in mean log fitness between parents and offspring of a cross, we found evidence for synergism between UV-induced mutations for both fitness parameters, K and r. The skewness test presented in this paper should be more robust, because it does not rest on the additional assumption of a rank-order relationship between mutation number and fitness that the earlier test made. Consistent with this notion is the fact that we could not unambiguously show synergism for K among accumulated mutations with the former test (DE VISSER *et al.* 1996), while we can with the skewness test. Furthermore, mutations induced by UV irradiation may have a different character than the slightly deleterious ones that are thought to predominate if accumulation is by Muller's ratchet (HAIGH 1978).

The difference in results for r and K should not be given too much emphasis, because the power of the skewness test will probably be different for the two parameters and because the negative correlation we observe between r and K indicates that they may not be fully independent measures of fitness. Speculatively, however, our results might imply that the advantage of sex provided by the Deterministic Mutation hypothesis (KONDRASHOV 1988) depends on situations of high population density. HAMILTON *et al.* (1990) predicted the relative importance of the Deterministic Mutation hypothesis in saturated environments, emphasizing the significance of truncation-like selection in such situations due to limited space or nutrients. Using arguments about metabolic pathways SZATHMÁRY (1993) predicted the prevalence of synergism if fitness is density dependent. These findings appear to be consistent with the observed ecological distribution of sexual species, which are more abundant in constant environments, where populations may reach high densities and selection on K predominates (BELL 1982; TRIVERS 1985). Data on deleterious mutation rate and epistasis between deleterious mutations in other species and with respect to other fitness estimates, related to different ecological settings, are needed to test the possible prevalence of synergism in saturated environments. We believe the skewness test presented in this paper provides a valuable tool for this purpose.

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