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Bias in the introduction of variation as an orienting factor in evolution

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SUMMARY According to New Synthesis doctrine, the direction of evolution is determined by selection and not by "internal causes" that act by way of propensities of variation. This doctrine rests on the theoretical claim that because mutation rates are small in comparison to selection coefficients, mutation is powerless to overcome opposing selection. Using a simple population-genetic model, this claim is shown to depend on assuming the prior availability of variation, so that mutation may act only as a "pressure" on the frequencies of existing alleles, and not as the evolutionary process that introduces novelty. As shown here, mutational bias in the introduction of novelty can strongly influence the course of evolution, even when mutation rates are small in comparison to selection coefficients. Recognizing this mode of causation provides a distinct mechanistic basis for an "internalist" approach to determining the contribution of mutational and developmental factors to evolutionary phenomena such as homoplasy, parallelism, and directionality.

INTRODUCTION

Molecular sequence comparisons reveal a variety of patterns of divergence suggesting the strong influence of mutation biases (Beletskii et al. 2000; Berg and Silva 1997; Cunningham et al. 1997; de Jong and Ryden 1981; Foster et al. 1997; Gojobori et al. 1982; Golding 1987; Gu et al. 1998; Macey et al. 1997; Schug et al. 1998; Wolfe 1989). A similar role has been suggested, more speculatively, for developmentally caused biases in variation (Arthur 1997; Thomson 1985). Surprisingly, although the process of variation has long been acknowledged as a mediator of "constraints" (in the sense that a mutationally or developmentally impossible change is an evolutionarily impossible change), a general causal principle by which the output of evolution reflects propensities of variation is missing from contemporary evolutionary theory.

The absence of such a principle is no mere oversight, but reflects a doctrinal position of the "New Synthesis." Prior to the development of the New Synthesis from 1930 to 1950, a great many evolutionists held that although the culling effect of Darwinian natural selection restricted possible outcomes, internal factors reflected in mutation and development supplied the initiative in evolution, and gave shape and direction to its course (Arthur 1997; Bowler 1988). Evolutionary change was seen as the cumulative result of transformations of development, and so it was natural to assume that internal propensities of development would influence the course of evolution. The New Synthesis view was distinguished from this "Old Synthesis" (Gilbert et al. 1996) by its utter denial of any internal causes of directionality, a position based on population-genetic arguments purporting to show that biases in variation cannot influence the course of evolution, because mutation rates are small in comparison to selection coefficients.

This view is challenged here. Conceptual arguments and computer-based analyses of a population-genetic model are used to advance the following points relating to bias in the process of individual variation as an orienting factor in evolution. First, the New Synthesis doctrine against internal causes of orientation lacks a valid basis: for practical purposes, the "shifting allele frequencies" paradigm defines "evolution" as the sorting out of pre-existing variation, so that mutation is treated only as a weak "pressure" on the frequencies of pre-existing alleles, and not as the origin of allelic novelty. Second, when both the novelty-introducing role of mutation and the allele-frequency-shifting role of selection are included in an evolutionary model, a bias in the introduction of novelty strongly biases the course of evolution, even when mutation rates are much smaller than selection coefficients. Third, this biasing influence represents a distinct and largely unappreciated mode of evolutionary causation. Case studies are presented to illustrate how bias in the introduction of variation may be expected to contribute to homoplasy, parallelism, and directionality. The recognition of this causal principle has broad implications for understanding the causes of non-randomness in evolution, and for clarifying both the mechanistic basis of an "internalist" approach to the study of evolution and its distinction from neo-Darwinism.

Mutation as a "pressure"

The New Synthesis position on the unimportance of biases in variation derives from the mutation/selection balance model

developed by R. A. Fisher (1930) and J. B. S. Haldane (1932). Fisher and Haldane showed that, starting with a population of *A1* individuals, recurrent mutation to an alternative allele *A2* will not result in fixation of *A2* if this is opposed by selection. Instead, given that mutation rates are small in comparison to selection coefficients, *A2* will persist at a low frequency, reflecting a balance between the opposing pressures of mutation and selection. In the simplest haploid case, the equilibrium frequency of *A2* is given by

$$f_2 \approx \frac{\mu}{s} \qquad (1)$$

where μ is the *A1*→*A2* mutation rate and *s* is the selection coefficient favoring *A1*.

Treating mutation and selection in this manner, as opposing pressures on allele frequencies, appeared to reduce the vexing issue of internal orienting factors to a simple matter of size. Since selection coefficients on the order of 10^{-2} or 10^{-3} were thought to be common, a general conclusion seemed warranted:

> The frequency of individual mutations in *Drosophila* is certainly seldom greater than one in 100,000 individuals, and we may take this figure to illustrate the inefficacy of any agency, which merely controls the predominant direction of mutation, to determine the predominant direction of evolutionary change (Fisher 1930).

The claim that because the rate of mutation is small, mutation is an ineffectual evolutionary force (see also Haldane 1933) was readily adopted by the New Synthesis architects as proof that internal causes of directionality had no valid basis (e.g., Huxley 1964; Mayr 1963; Simpson 1967; Stebbins 1966).

Although the issue of internal orienting factors has receded from the attention of evolutionary biologists, the "opposing pressures" view of mutation and selection remains. The claim that mutation "pressure" is an ineffectual evolutionary force is repeated in textbooks (Freeman and Herron 1998), applied in contemporary research (Bull et al. 1997), and cited in authoritative reviews (Leigh 1987; Maynard Smith et al. 1985; Sober 1987). The proposition that mutation is not a significant orienting factor in evolution is seen as an important theoretically based conclusion, as indicated in a recent statement on the scientific status of evolutionary theory (Futuyma et al. 1998). Whereas neutralists would dispute the applicability of this conclusion at the molecular level, both neutralists and selectionists treat mutation and selection as opposing pressures (Gillespie 1991; Gu et al. 1998), while disagreeing on whether selection "pressure" is ever sufficiently weak to allow mutation "pressure" the upper hand. Yet, mutation has a unique role as the origin of novelty that is not reflected in this conception of evolutionary causes.

Mutation as the origin of novelty

The distinction between mutation as a "pressure" that might cause fixation of an allele, and mutation as the ultimate origin of allelic novelty, is commonly made in introductory treatments of evolutionary mechanisms (e.g., Arthur 1984; Freeman and Herron 1998). As a "pressure," mutation may shift the relative frequencies of alleles that are already present in a population, subtracting from one frequency and adding to another, but such effects are swamped by those of selection (and drift), given that mutation rates are so small. As the origin of allelic novelty, however, nothing can replace mutation. The latter point is not apparent if one defines evolution as "shifting allele frequencies" and then identifies the "forces" of evolution as mutation, selection, drift, and migration (Stebbins and Ayala 1981). This conception fails to indicate that only one of the "forces" may shift an allele frequency upwards from zero (e.g., from 0 to 1/N), and that this distinct step is a necessary precursor to the shifting effects of any of the other "forces."

The value of distinguishing the introduction of novelty as a distinct step in the evolutionary process may be illustrated by considering the case of mutation-biased neutral evolution, the basis of which is clear from stochastic population-genetics theory. Since the rate of mutational introduction of neutral alleles is $N\mu_n$ per generation (μ_n, neutral mutation rate per individual per generation; N, population size), and the probability of random fixation is 1/N for a newly introduced allele, the steady-state rate of neutral origin-fixation (Kimura 1983) is:

$$R_n = (N\mu_n)\frac{1}{N} = \mu_n \qquad (2)$$

From this, it follows that a bias in mutation rates $\mu_1/\mu_2 \neq 1$ is a bias on rates of neutral evolution (i.e., $R_1/R_2 = \mu_1/\mu_2$).

Although this effect is typically described as a matter of directional mutation "pressure" (e.g., Gautier 2000; Sueoka 1988), this terminology is misleading. Indeed, the "opposing pressures" metaphor does not even apply to neutral evolution: for mutation-biased neutral evolution, it is not necessary for mutation rates to exceed selection coefficients, but only that the latter be sufficiently small (i.e., $s << 1/N$). When mutation-biased neutral evolution occurs, this is due to a bias in the origin process, not a bias in the fixation process, as one may gather by considering that in Equation 2 above, the mutation rate does not appear in the term for probability of fixation (i.e., 1/N), only in the term for the rate of origin (i.e., $N\mu$).

Combining a biased origin process and a selective fixation process

The basis for a general theoretical conclusion about the possible orienting role of mutation bias does not apparently exist. The classic Fisher-Haldane argument demonstrates only that mutation is not an effective surrogate for selection as a cause of allele fixation, and fails to address any implications of the novelty-introducing role of mutation. Although the neutral model suggests that this novelty-introducing role is

crucial, the question remains as to how effective a bias in the origin process would be in a non-neutral model.

This issue may be addressed simply by including a biased origin process in a model of selective allele fixations. The model must allow for at least two types of alleles that are initially absent, so that a bias in their rates of introduction may be imposed. At minimum, then, one may consider a haploid two-locus model in which an initial population of *ab* individuals is subject to mutations that introduce genotypes *Ab* and *aB* at two different rates. For fixations to occur, these mutations could be either neutral or beneficial, but since our concern is to address selective allele fixations, they must be beneficial. Of interest is the non-trivial case in which the alternative genotype of relatively higher fitness is introduced by mutation at the relatively lower rate. Choosing *Ab* arbitrarily as the alternative genotype of higher fitness, the fitnesses of *Ab* and *aB* would be $1 + s_1$ and $1 + s_2$, respectively, with $s_1 > s_2 > 0$, whereas the mutation rates (from *ab*) would be μ_1 and μ_2, respectively, with $\mu_1 < \mu_2$, as shown in Figure 1.

Whether mutation can bias the course of evolution in this model depends on other conditions, the significance of which can be summarized by referring to an idealized case in which mutation biases will have no impact. In general, for any multilocus model, one expects that mutational biases will have no substantial influence if (1) fitness effects are independent and (2) the equilibrium state is taken to be the definitive outcome (i.e., no matter how long it takes to reach it).

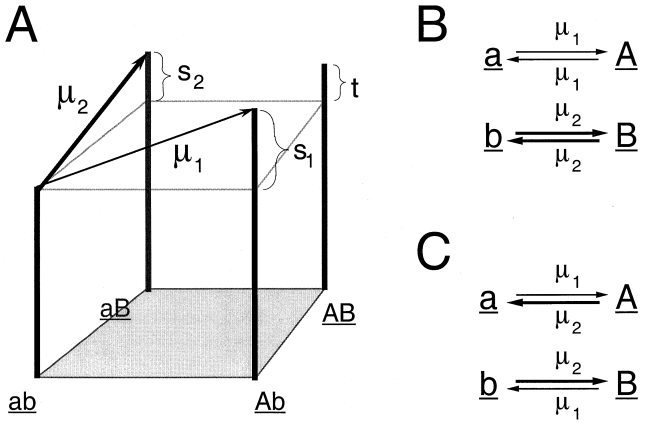

Fig. 1. Evolutionary model. (A) Relationships of parameters are indicated graphically in "fitness landscape" form, where the horizontal axes represent discrete genotypic values, and the vertical axis represents individual fitness. Fitnesses for *ab*, *Ab*, *aB* and *AB* are 1, $1 + s_1$, $1 + s_2$, and $1 + t$, respectively, with $0 \leq t \leq s_2 < s_1$. Rates of forward mutation from *a* to *A* and *b* to *B* are μ_1 and μ_2, respectively, with $\mu_1 < \mu_2$. Rates of reverse mutation (which, in practice, make little difference) may be assigned according to one of two models: (B) the mutation rate is higher for the interconversion of *b* and *B* than for that of *a* and *A*; or (C) a forward/reverse bias favors $b \rightarrow B$ and $A \rightarrow a$ over the reverse mutations.

Under such conditions, mutation might bias the initial course of evolution, but there will always be a path to the fitness optimum, which will always be reached at equilibrium, so that the net course of evolution will be predictable from fitness effects alone. Under any other assumptions, mutation biases will be important to the extent that they influence which origin-fixation events occur first. If there are allele-dependent interactions between loci, then mutation may bias the choice of local fitness peaks by biasing for which origin-fixation steps occur first. Likewise, even given independent fitness effects, one need not give credence to the notion of an equilibrium outcome, since there is no natural lower limit on the rate of mutational introduction of beneficial variants. For any finite interval of divergence, presumably there are always possible variants whose cumulative probabilities of mutational introduction are sufficiently close to zero for non-uniformity in mutation rates to be important.

For any such reason, it is of interest to address which of two origin-fixation events will occur first. To focus on this issue in the model outlined above, one may simply assign *AB* a fitness lower than that of either *Ab* or *aB* (Fig. 1A) so that the initial *ab* population will evolve to one of two isolated fitness peaks (*Ab* and *aB*), but will not cross from one to the other nor proceed to *AB*. The question of "direction" is then a simple matter of whether the allele fixation is of *A* or *B*. This fitness scheme is an instance of the classic Bateson-Dobzhansky-Muller model of speciation (Orr 1996), in which variant alleles that are separately beneficial, but deleterious in combination, are fixed in isolated sub-populations, with the result that a subsequent fusion of the populations is prevented by the decreased fitness of hybrids.

ANALYSIS OF AN EVOLUTIONARY MODEL

The haploid Bateson-Dobzhansky-Muller model (Fig. 1) was implemented as an individual-based Monte Carlo simulation (using software available from the authors). Haploid individuals in a population were subject to mutation, recombination, and reproduction (in that order) in each generation. Population sizes varied from 10^2 to 10^6. Generally 400 or more replicates were done for each set of conditions (except for the largest population size). Mutation rates follow the fully reversible model (Fig. 1B), but results for the forward/reverse model (Fig. 1C) are not significantly different (not shown). Likewise, the value of t (here, $t = 0$) is relatively unimportant and is not discussed further.

Of interest for a given set of conditions is the bias in outcomes, that is, the number of times the population evolves to the mutationally favored peak (*aB*), divided by the number of times it evolves to the peak of highest fitness (*Ab*). For convenience, the inequality in fitness of *Ab* and *aB* is defined

as a bias in selection coefficients, $K = s_1/s_2$, with the bias in mutation defined oppositely as $B = \mu_2/\mu_1$.

Effects of B, N, K and s

Figure 2A shows the bias in outcomes as a function of mutation bias $B = \mu_2/\mu_1$, where the selection coefficients are $s_1 = 0.02$ and $s_2 = 0.01$ (i.e., $K = 2$) and given free recombination ($R = 0.5$). Wherever the bias in outcomes exceeds unity, the majority outcome is *aB*, and thus it may be said that mutational bias determines the predominant direction of evolutionary change, a result seen for all the population sizes shown. In general, there is an obvious positive correlation between bias in outcomes and bias in mutation. This effect is not due to mutation "pressure" in the sense of high mutation rates: in all of these simulations, μ_2 (the higher mutation rate) is held constant at a value at least 100-fold smaller than s_2 (the smaller selection coefficient). Reducing the rate of recombination has little effect. Even the entire absence of recombination (Fig. 2B) has little effect when $N\mu << 1$, and otherwise, it reduces but does not eliminate the ability of mutation to bias the course of evolution.

For a large region of parameter space, the effects of both B (mutation bias) and K (selection coefficient bias) are linear on the bias in outcomes. This relationship is shown in Figures 2 and 3 by the dashed line, which is $B/K = \mu_2 s_2/(\mu_1 s_1)$. For the conditions in Figure 2, the bias in outcomes is not significantly different from B/K for $N = 10^2$ (Fig. 2, diamonds) and $N = 10^3$ (not shown); the bias in outcomes is somewhat less than B/K for $N = 10^4$ (Fig. 2, triangles), and several-fold less for $N = 10^5$ and $N = 10^6$. Likewise, as shown in Figure 3, increasing K reduces the bias in outcomes, but comparable increases in B counteract this effect. Finally, the magnitude of selection coefficients can be varied considerably with little effect on outcomes (Fig. 4).

Stochastic approximation for small Nμ

The tendency for the bias in outcomes to coincide with the B/K ratio can be explained with reference to stochastic pop-

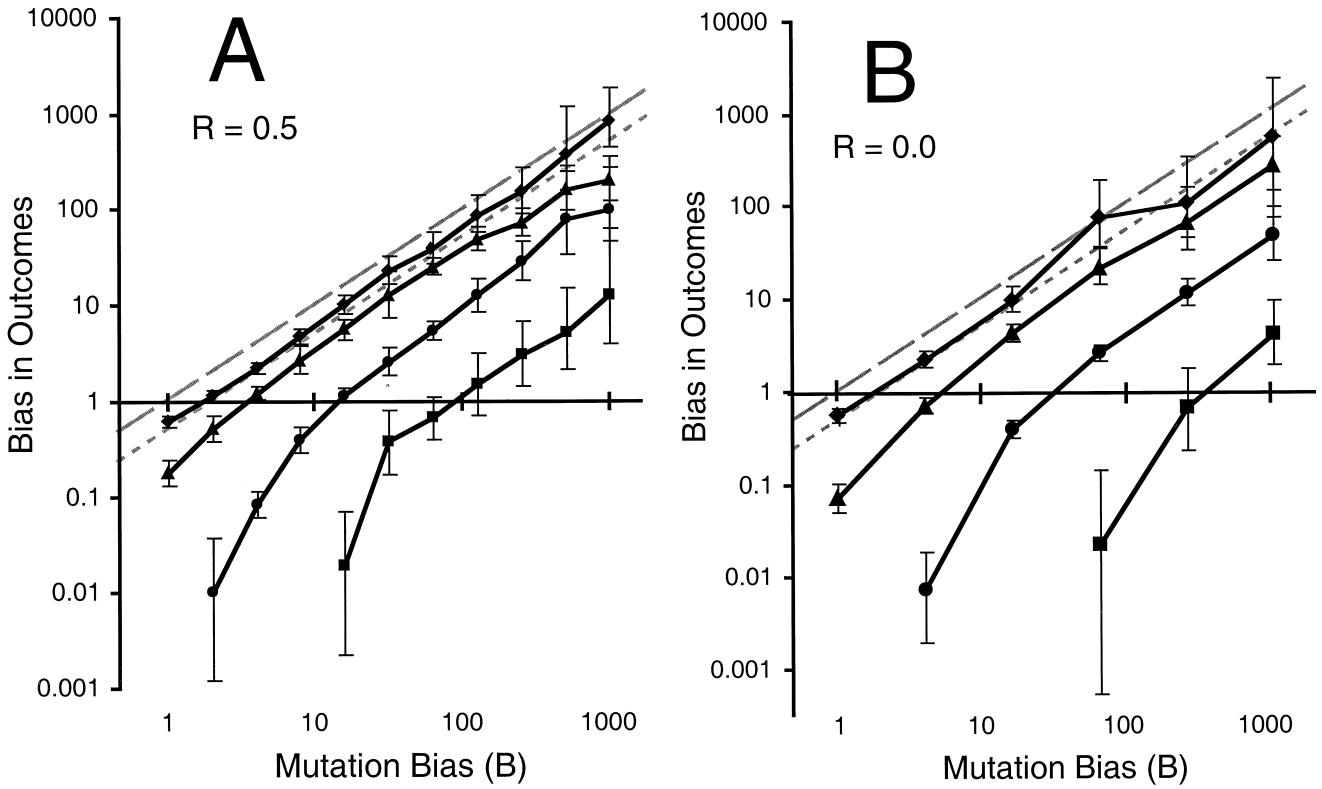

Fig. 2. Bias in outcomes as a function of mutation bias, for four population sizes. Results in (A) for the case of free recombination ($R = 0.5$), and in (B) for the case of no recombination ($R = 0$), are shown for population sizes of 10^2 (diamonds), 10^4 (triangles), 10^5 (circles), and 10^6 (squares). Selection coefficients are $s_1 = 0.02$ and $s_2 = 0.01$ (thus $K = 2$). The higher mutation rate is fixed at $\mu_2 = 10^{-5}$, whereas the lower rate varies over three orders of magnitude according to the value of $B = \mu_2/\mu_1$ shown on the horizontal axis. For population sizes of 10^2 to 10^4, the bias in outcomes is close to B/K (short dashes, in grey), with or without recombination. Since selection is significant, the results differ from the neutral expectation of $\mu_2/\mu_1 = B$ (long dashes, in grey). Vertical bars represent the standard error, calculated from the binomial error following Zar (1999).

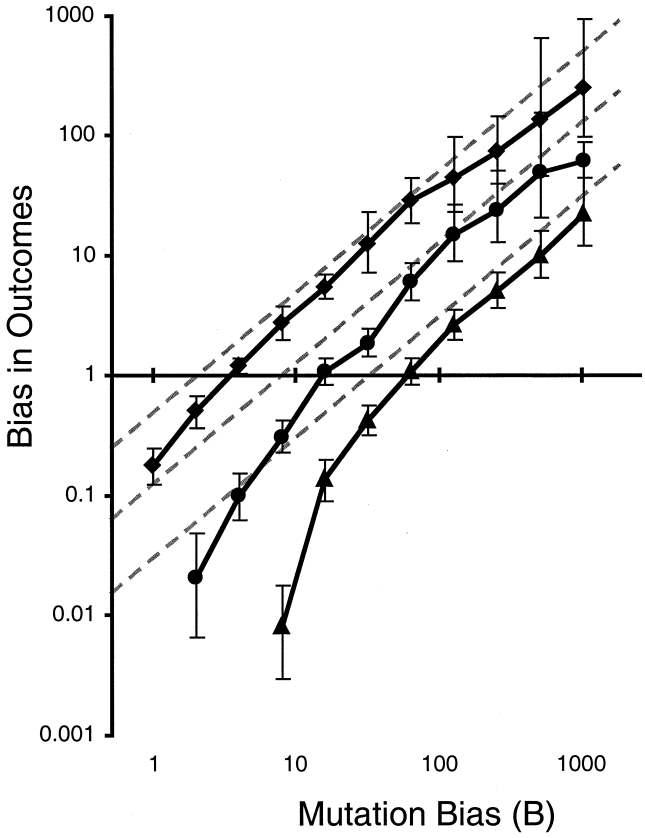

Fig. 3. Effect of K. Bias in outcomes is shown as a function of mutation bias, for K = 2 (diamonds), K = 8 (circles), and K = 32 (triangles), with the larger selection coefficient held constant, s_1 = 0.02, where $\mu_2 = 10^{-5}$, N = 10^4, and R = 0.5. Increasing K decreases the bias in outcomes, but this effect can be overcome by larger biases in mutation, the two effects being approximately linear as B becomes moderately large, as shown by the relationship to B/K (dashed lines).

ulation genetics theory. Due to stochastic variance in reproduction, a newly arising beneficial allele is likely to be lost within a few generations, the probability of fixation being only about 2s (Kimura 1983). Given a rate of introduction of $N\mu_b$ beneficial alleles per generation, the steady-state rate of origin-fixation is then

$$R_b \approx 2N\mu_b s \quad (3)$$

When the outcome of evolution is a matter of which of two origin-fixation events occurs first, as it is here, then the bias in outcomes will approach the ratio of rates

$$\frac{R_2}{R_1} \approx \frac{2N\mu_2 s_2}{2N\mu_1 s_1} = \frac{\mu_2 s_2}{\mu_1 s_1} = \frac{B}{K} \quad (4)$$

to the extent that the two possible origin-fixation events occur independently. This assumption of independence will be violated to the extent that *Ab* and *aB* genotypes are present in the population at the same time, which will often be the case when $N\mu \geq 1$. Thus, when $N\mu << 1$, the bias in outcomes expected from stochastic origin-fixation kinetics is simply the B/K ratio (Eq. 4). This expected relationship is shown more clearly in Figure 5, which provides yet another demonstration that the biasing influence of mutation shown here is not a matter of "pressure": when $N\mu$ is not already small, *decreasing* the rate of mutation actually *increases* the effect of the mutation bias.

Dependence on initial allele frequencies

Finally, to clarify the reasons that the results presented here support a heterodox conclusion, it is helpful to demonstrate how these results are entirely dependent on the heterodox assumption that initial variation is absent. The introduction of a new allele by mutation is traditionally seen as a precondition for evolution, rather than as part of the evolutionary process itself:

> Evolution is not primarily a genetic event. Mutation merely supplies the gene pool with genetic variation; it is selection that induces evolutionary change. (Mayr 1963)

That is, the process of "evolutionary change" is seen to begin with the process of fixation, not with the introduction of a new allele.

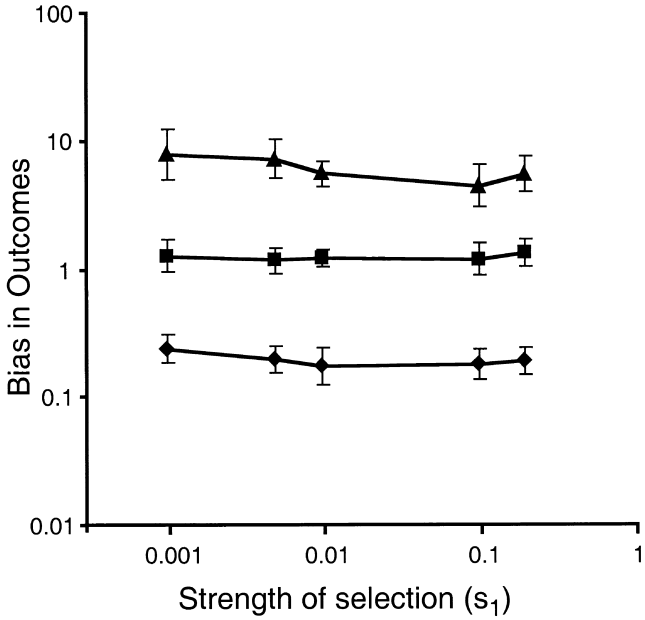

Fig. 4. Effect of the magnitude of selection coefficients. Bias in outcomes is shown as a function of the higher selection coefficient, s_1, for B = 16 (triangles), B = 4 (squares), and B = 1 (diamonds), where K = 2, $\mu_2 = 10^{-5}$, N = 10^4, and R = 0.5. Varying the magnitude of selection coefficients 200-fold from s_1 = 0.001 to s_1 = 0.2, with a constant bias in selection coefficients (K = 2), has little effect on the bias in outcomes. Vertical bars are the standard error calculated as described for Fig. 2.

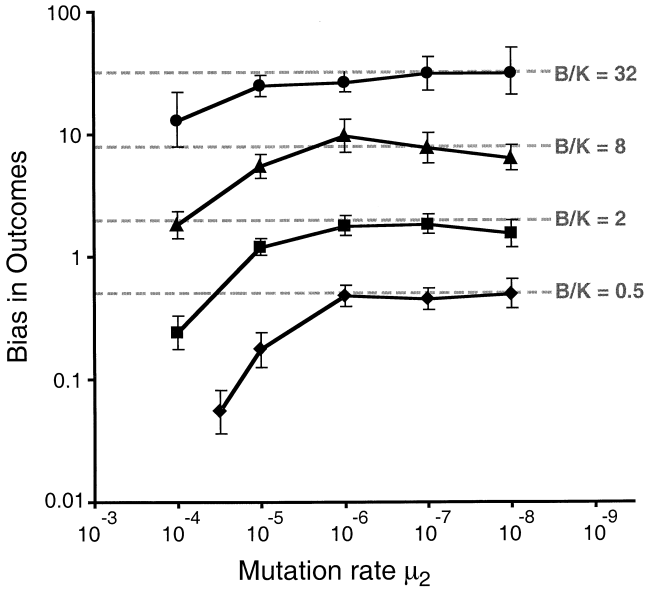

Fig. 5. Convergence on B/K as Nμ becomes small. Bias in outcomes is shown as a function of μ_2, *decreasing* from left to right, for B = 64 (circles), B = 16 (triangles), B = 4 (squares), and B = 1 (diamonds), where $N = 10^4$, $s_1 = 0.02$, $s_2 = 0.01$ (i.e., K = 2), and R = 0.5. Starting at the left with $\mu_2 = 10^{-4}$, the value of Nμ is not small (Nμ = 1), and the bias in outcomes is considerably less than B/K. As μ_2 decreases, the value of Nμ becomes very small (Nμ = 0.0001 when $\mu_2 = 10^{-8}$) and the bias in outcomes becomes indistinguishable from B/K. Vertical bars are the standard error calculated as described for Fig. 2.

To invoke a "gene pool" thus, as a kind of intermediary between the novelty-introducing process and "evolution" (see also Stebbins 1966), might seem to be an empty verbal argument in some contexts, but in the context of constructing an evolutionary model, it clearly favors the assumption that the allelic variation relevant to "evolution" is already present in the initial population. The effect of this assumption is illustrated in Figure 6. When alleles *A* and *B* are present initially, even at low frequencies of 0.005, mutation bias is utterly ineffective in orienting evolution (Fig. 6), unless mutation rates are allowed to grow unreasonably large, creating a substantial effect of "pressure" (not shown). Thus, when the prior existence of variation is taken for granted, "evolution" is defined in such a way that mutation cannot act as the origin of novelty, and consequently, the behavior of the system is as expected from the "opposing pressures" metaphor.

APPLICABILITY TO EVOLUTIONARY PROBLEMS

In a model of evolution to one of two isolated fitness peaks, a bias in mutation rates has a strong orienting effect. This effect is not merely to result in the occasional chance fixation of the mutationally favored, but relatively less advantageous, genotype. Instead, there is always some value of mutation bias sufficient to orient evolution predominantly toward the mutationally favored outcome.

Whether such biases might contribute importantly to the course of evolution in nature depends crucially on population sizes and mutation rates. The effect of a bias in mutation is strongest when mutations are rare, $N\mu << 1$, though strong effects are still seen so long as $N\mu \leq 10$. Population sizes for multicellular organisms studied by population biologists are usually in the range 10^3 to 10^9 (Nei and Graur 1984; Nevo et al. 1984) whereas cosmopolitan microbes may have far larger population sizes, such as 10^{20} for *E. coli* (Nei and Graur 1984) or even 10^{27} for the ubiquitous marine cyanobacterium *Prochlorococcus* (E. Urbach, pers. comm.). Experimentally determined average rates for point mutations in cellular organisms are 10^{-8} to 10^{-10} per base-pair per organismal generation, the higher values applying to multicel-

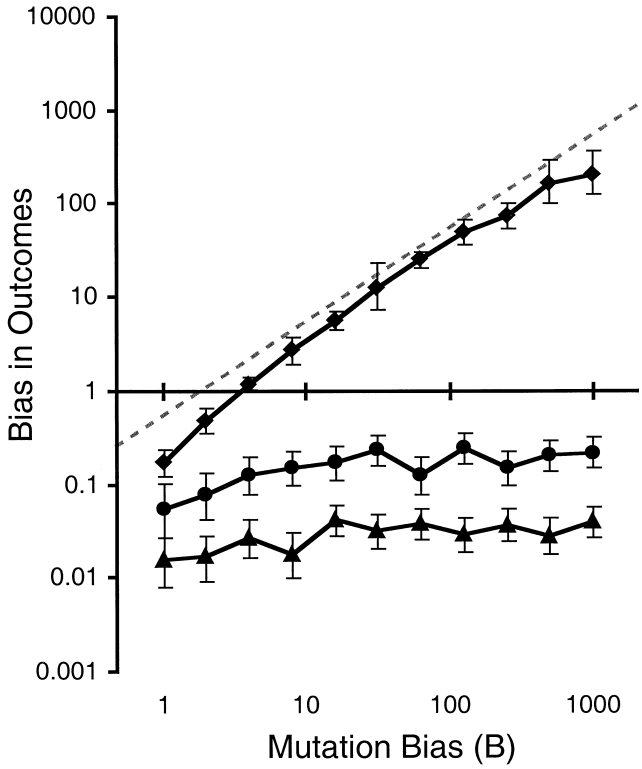

Fig. 6. The effect of initial variation. Bias in outcomes is shown as a function of mutation bias for three different initial frequencies of alleles *A* and *B*, p = 0 (diamonds), p = 0.005 (circles), and p = 0.01 (triangles), where $\mu_2 = 10^{-5}$, $N = 10^4$, $s_1 = 0.02$, $s_2 = 0.01$ (i.e., K = 2), and R = 0.5. For p = 0, the series of results shown is the same as that for $N = 10^4$ in Fig. 2A, and the effect of mutation bias is strong (compare to B/K, dashed line). For p = 0.005 and p = 0.01, increasing mutation bias over three orders of magnitude has almost no effect on the bias in outcomes. Vertical bars are the standard error calculated as described for Fig. 2.

lular organisms with many cell generations per organismal generation (Drake et al. 1998). Thus, it would appear that $N\mu \leq 10$ for point mutations in most multicellular organisms, as well as in microbes with population sizes less than 10^{11}.

Average mutation rates, such as those cited above, invariably encompass a wide range of individual rates for different mutational routes and different genetic sites (see literature cited by Drake et al. 1998). Site-to-site differences in rates of point mutation of two orders of magnitude or more are common, as was apparent even in the early results of Benzer (1961).

Only a few modest non-uniformities among mutational routes have been studied in detail. For instance, purine-to-purine and pyrimidine-to-pyrimidine nucleotide substitutions, called "transitions," typically occur at a rate several-fold higher than that of "transversions" (Bell et al. 1997; Schaaper and Dunn 1991). These rates are further influenced by neighboring nucleotides (Blake et al. 1992), the strongest effect being the presence of a methylatable CpG dinucleotide (Krawczak et al. 1998). So-called "GC bias" favors mutation from A or T nucleotides to G or C nucleotides (Cox and Yanofsky 1967; A, adenine; G, guanine; C, cytosine; T, thymine). With respect to the two-locus model explored above, the influence of a transition/transversion bias of degree $B = \mu_2/\mu_1$ can be understood as a case of the fully reversible mutation model (Fig. 1B) with *b* and *B* interconverted by a transition, *a* and *A* by a transversion. The influence of a GC bias of $B = \mu_2/\mu_1$ can be understood according to the mutation model in Figure 1C, with $b \rightarrow B$ representing an AT→GC change, and $a \rightarrow A$ a GC→AT change.

Thus, mutation biases may be expected to exert some degree of influence on the course of evolution in the many natural circumstances in which such biases exist and $N\mu$ is not overly large. To lend a more concrete significance to this possibility, the role of bias in the introduction of variation is discussed below with respect to three areas of current interest in evolutionary biology.

GC/AT bias and amino acid composition

The composition of proteins encoded by genomes rich in G or C nucleotides is biased toward amino acids with GC-rich codons (Singer and Hickey 2000). The interpretation of such results has been dominated by the "opposing pressures" metaphor and the neutralist-selectionist debate: directional change in GC content is attributed either to selection "pressure" for a higher GC content (Bernardi et al. 1997; Eyre-Walker 1999; Gillespie 1991) or to neutral evolution biased by GC/AT mutation "pressure" (Gu and Li 1995; Sueoka 1988).

However, as shown above, mutation-biased evolution and selective allele fixations are not mutually exclusive. The manner in which they might operate together may be illustrated by considering the problem of increasing the stability of a protein by filling cavities in its interior (Eriksson et al. 1992; Liu et al. 2000). A typical cavity might be filled by replacing one of the amino acids abutting the cavity with a bulkier amino acid. Although several such cavity-filling replacements might be possible for a given cavity, once the first has occurred, further replacements would be deleterious (an interaction that results in roughness of fitness landscapes). Thus, since cavities are filled on a "first come, first served" basis, the process may be adaptive by virtue of increasing stability, and at the same time, biased by mutation.

Considering the overall effect of such a process in some species, a global increase in protein stability could occur due to natural selection, with a concomitant global shift in GC-content (and amino acid usage) due to mutation biases. The amino acid exchanges favored by a mutation bias, though they may be adaptive, need not be the most advantageous possible changes in order to be favored by the overall origin-fixation process (as shown above), thus there need not be a global positive correlation between protein stability and amino acids with GC-rich codons. The same kind of mutation-biased adaptation could result for some other aspect of protein fitness (i.e., other than thermostability), as long as the relevant fitness landscapes are "rough" due to interactions between amino acid sites.

Parallelism in bacteriophage evolution

Although mutation-biased neutral evolution remains a viable hypothesis for the case of GC-content and a great many other sequence characteristics subject to directional changes (see Stoltzfus 1999 and references therein), neutral evolution sometimes can be ruled out. In recent reports of parallel evolution in laboratory populations of bacteriophage ϕX174, the observed dynamics of population change indicate that most fixations are not due to drift (Bull et al. 1997; Wichman et al. 1999). Bull and colleagues (1997) address the causes of parallelism thus:

> Mutation pressure alone is unlikely to cause a meaningful increase in substitution frequency in our design. At 1000 doublings, a mutation rate of 10^{-5} per doubling would lead to a final frequency of only 0.01, not enough to observe in multiple lineages. Consequently, the plausible alternative is selection.

This conclusion, based on the inadequacy of mutation "pressure" to cause allele fixations, leaves the impression that population-genetic reasoning excludes a contribution of mutation to parallel evolution. Yet, a contribution of mutation bias, specifically a transition/transversion bias, is suggested forcefully by the data. Considering only the amino acid replacements that occurred in parallel in two or more lineages, there are 28 types of replacement events, each of which occurred three times, on average, for a total of 84 changes (based on results from Table 1 of Bull et al. 1997, and sequence data kindly provided by H. Wichman). For these 28 types of event, the ratio of transitions to transversions is 21/7, significantly higher ($X^2 = 22$) than the value of 0.5 expected from the assumption of rate uniformity (given that there are twice as many possible transversions as transi-

tions), but not significantly different ($X^2 = 2.6$) from the ratio of 1.5 expected from studies of mutation in the bacterial host, *E. coli* (Bell et al. 1997; Schaaper and Dunn 1991).

Parallelism is more likely the fewer the possible changes, and the less uniform their probabilities. Selection may be said to act in both ways, effectively limiting the possible changes to those that are beneficial or neutral, and modulating the probability of fixation of the beneficial changes (strictly speaking, these are not two effects, but one effect of probability, since deleterious alleles also have non-zero fixation probabilities). However, there is no reason to believe that there exist "constraints" that limit possible mutations by preventing transversions. Presumably, transversion mutations simply occur at lower rates than transitions, and this non-uniformity contributes to parallel evolution by way of a bias in the introduction of novel beneficial alleles.

Two further aspects of this case deserve comment. First, it is not necessary to posit epistasis (leading to rough fitness landscapes) to explain a strong influence of mutation bias. Because the organisms in these experiments have been exposed to new conditions, the short-term outcome of evolution may be dominated by the kinetics of origin-fixation. Second, this case highlights the need for a somewhat subtle distinction between directionality and relative orientation. Although a transition/transversion bias may contribute to homoplasy and parallelism, this kind of bias can not be the efficient cause of cumulative change in a property of state: increasing the fully reversible rate of interconversion of C and T, and of A and G, will not result in a net change in nucleotide composition. By contrast, a non-uniformity in forward and reverse rates, such as GC/AT bias or deletion/insertion bias, may result in a cumulative directional change in some property, such as GC content or sequence length, respectively (Petrov et al. 2000; Sueoka 1988).

Developmental biases in variation

Biases in the rates of mutation between specific pairs of genetic states, such as those depicted in Figures 1B and 1C and referred to here as *mutation biases*, are not the only conceivable source of bias in the introduction of variation. Even in the (purely hypothetical) case of no mutation bias, one would still expect non-uniformity among mutant phenotypes, with some phenotypes arising more commonly than others. A variety of observations suggest the importance of such *developmental biases* in variation, the most familiar being the many instances of "phenocopies," phenotypes that are characterized initially as the result of some specific mutation but that are discovered subsequently to result from entirely different mutations or, in the absence of mutation, from altered conditions of growth. An acute demonstration of evolutionarily important developmental biases is provided Alberch and Gale (1985), who found an apparently strong correlation between evolved differences in amphibian limb morphology, and taxon-specific propensities in the altered limb morphologies that result from experimental perturbation of limb development. A more general source of evidence for the pervasive presence of developmental biases in variation (though not by itself a source of evidence for their evolutionary importance) is the considerable empirical support for Cheverud's conjecture of a correspondence between genetic and non-genetic components of co-variation in phenotypic characters (e.g., Waitt and Levin 1998). Observations such as these all point to the existence of developmental operators that respond asymmetrically to perturbation, including genetic perturbation.

Whether developmental biases may be expected to exert an orienting influence is an issue that can be addressed by reference to the results already presented. To do so requires some mapping of genotypes to phenotypes that reflects developmental processes. An explicit model of development is not actually necessary if this mapping embodies the relevant principle of development. In the present instance, the only principle that we wish to embody is that some alternative phenotypes are developmentally favored such that they can arise by many different mutations (e.g., as suggested by Alberch and Gale 1985), or more abstractly, that there are non-uniformities in the representation of phenotypes in a local (mutational-accessibility-weighted) region of genotype space. Thus, if phenotypes I, II, III, and IV develop from genotypes *ab*, *Ab*, *aB*, and *AB*, respectively, then to represent phenotype III as a developmentally favored phenotype, one may suppose that mutations at, not just one locus, but a total of L loci (i.e., $b_1 \rightarrow B_1$, $b_2 \rightarrow B_2$, $b_3 \rightarrow B_3 \ldots b_L \rightarrow B_L$), give rise to phenotype III. All of these, because they exhibit phenotype III, may be assumed to exhibit the negative fitness interaction with *A*.

The outcome of such a model is obvious. With no bias in rates of mutation, there is an L-fold developmental bias favoring the introduction of phenotype III. Assuming free recombination, the influence of such a bias will be the same as that of a mutational bias of magnitude B = L. Under lesser degrees of recombination, assumptions about the map locations of the various loci would be required, yet would seem unimportant, since the degree of recombination has little effect (above). If so, then the orienting effect of a developmental bias will be the same as that of a mutation bias of the same degree.

SYNTHESIS

As a theoretically distinct evolutionary cause of orientation, bias in the introduction of variation has the same formal status as natural selection. A cause-and-effect relationship is established from the results given here in that non-randomness in mutation has a predictable effect on the outcome of an evolutionary process. This *predictable* effect may be said to

represent an *orienting*, *directional*, or *shaping* influence to the extent that a bias in evolutionary outcomes will be positively correlated with the bias in mutational outcomes when the biases are measured on the same scale. For instance, if the model of mutation is GC bias, then the scale is in units of increase in GC-content per unit time, and the greater the mutation bias, the more the outcome of evolution will be biased toward an increase in GC-content over time. Finally, this cause of orientation is distinct from natural selection in that (1) in any given case, it may favor a different outcome than that favored by selection; and (2) an orienting influence is expected whether allele fixations are selective (above) or random (Eq. 2). In short, bias in the introduction of novelty by mutation is a prior bias on the course of evolution, and may be said to be an "internal" cause of orientation or directionality. The same principle may be extended, under conditions outlined above, to the case of developmental biases in phenotypic variation.

The demonstration of this causal principle suggests the need for a broader synthetic view of evolutionary causes. A cause-and-effect relationship between internal propensities of variation and directions of evolutionary change was ruled out by the New Synthesis architects and, indeed, makes little sense in terms of the "shifting allele frequencies" conceptualization of evolutionary causes. A more general conceptual framework developed by Vrba and Eldredge (1984) provides a suitable basis for recognizing the introduction of novelty by mutation and development as a distinct evolutionary process. In their terms, evolution is a process of the *introduction and sorting of variation in a hierarchy of reproducing entities*. Sorting refers to changes in representation of variants that result from differential reproduction, either biased (selection) or unbiased (drift). Non-randomness in the course of evolution arises by one of two primary causes: bias in the introduction of variation, and bias in the sorting of variation. The results presented here clarify this view in two respects, namely that (1) quantitative biases in the introduction of variation (i.e., matters of degree, not just absolute "constraints") are effective; and (2) outcomes of evolution may be biased simultaneously by selection and by bias in the introduction of variation (i.e., mutation-biased evolution does not require neutral allele fixations).

The significance of recognizing bias in the introduction of variation as a distinct mode of causation is not that it provides the definitive answer to some clearly stated, pre-existing problem. Instead, it provides the basis for reformulating a variety of problems that have arisen in evolutionary biology since the New Synthesis, in a manner that clarifies issues of causation at the level of population genetics. These problems can be grouped into three categories, addressed below.

Distinguishing neo-Darwinism

The persistence of the neo-Darwinian view in the face of more than a century of adverse criticism has been interpreted by defenders as a sign of its strength, and by detractors as a sign of deeply ingrained prejudices. It might be argued instead that this persistence reflects the lack of clear contrast between, on the one hand, a version of neo-Darwinism that is committed to some claim of explanatory sufficiency (Saunders and Ho 1982), and on the other, an alternative view that differs substantively and that (1) can be expressed in similar terms, unlike structuralist or "evo-devo" alternatives that eschew population genetics (Gilbert et al. 1996), and (2) represents a general view of evolutionary causes, unlike the neutral theory (in the sense that it is offered as a theory specific to evolution at the "molecular level"; Kimura 1983), or views that invoke some cause (e.g., symbiogenesis, molecular drive) with a restricted domain of influence.

Within the conceptual framework outlined above, the neo-Darwinian view (as defined in the New Synthesis) emerges as a specific hypothesis about the causes of non-randomness. In this view, because an abundance of "random" variation can be taken for granted, non-randomness in the outcome of evolution is the consequence of biased sorting, that is, a consequence of fitness differences. The only recognized exception to the sufficiency of selective explanations is that "constraints" on variation are responsible for the non-occurrence of impossible changes. Otherwise, when one *possible* evolutionary change occurs more readily than another *possible* one, this can only be due to fitness effects. Such a view could prove false. A specific alternative is that the course of evolution has been strongly influenced by bias in the introduction of variation, such that homoplasy, parallelism, and directional trends cannot be explained solely by invoking fitness effects and impossibilities ("constraints").

Molecular evolution and neutralism

Within the framework presented above, neutralist explanations that invoke differential "selective constraints" actually have the same basis as neo-Darwinian ones, in that non-randomness in evolution is attributed to fitness effects (e.g., as in the prediction that the rate of synonymous codon changes will be higher than that of non-synonymous changes). However, other neutralist arguments rely on mutation biases to account for non-randomness, as in the prediction that sequences will become shorter due to a deletion/insertion bias in mutation (Petrov et al. 2000), or that amino acid composition will be shifted by a GC/AT bias in mutation (Sueoka 1988).

Evidence for the role of mutation biases in such cases should be reconsidered separately from the neutralist-selectionist controversy. Mutation biases have been invoked to account for non-randomness in a great variety of cases cited earlier. However, recognition of the importance of mutation biases has been held hostage to doubts about neutral evolution, on the assumption that mutation-biased evolution must involve neutral allele fixations. To recognize bias in the introduction of variation as a distinct evolutionary cause is to

negate this assumption, removing the obligation to resolve the neutralist-selectionist dispute before accepting (at least provisionally) the overwhelming *prima facie* evidence for an orienting role of mutation bias in molecular divergence.

Evolutionary developmental biology and the internalist view

The research strategy currently favored by evolutionary developmental biologists is a retrospective one of inferring the historical chronicle of molecular and developmental changes underlying phenotypic evolution, and of interpreting this chronicle in terms of concepts such as "duplication," "dissociation," and "self-organization" (e.g., Gerhart and Kirschner 1997).

Whether this research strategy reflects an underlying view of evolution that departs substantively from neo-Darwinism, or instead reflects some shift in perspective (i.e., a metaphysical or epistemological difference), is unclear. Contemporary biologists representing the internalist tradition (e.g., Gilbert et al. 1996; Goodwin 1994; Lauder 1981) have purported to explore laws that govern evolutionary change while denying any direct or necessary connection to population genetics theory, the mechanistic formalization of the evolutionary process that is the foundation of neo-Darwinism. Thus, Gilbert and colleagues (1996) write that the problems of interest in the nascent "evo-devo" synthesis are "not seen as being soluble by population genetics". Meanwhile, neo-Darwinian conservatives suggest that structuralists and "evo-devo" enthusiasts mistake mechanisms of development for mechanisms of evolution (e.g., Maynard Smith 1983; Mayr 1994). In their view, the principles of evolution are already known, and the evolutionary influence of development (like that of ecology or biochemistry) is a matter of selection acting on genes with developmental (or ecological or biochemical) effects (e.g., Maynard Smith 1998).

This situation is immediately clarified if one supposes that bias in the introduction of variation is the causal principle that, by its likely importance in evolution, and by its absence from neo-Darwinism, justifies an alternative, internalist view. If so, this view would be distinguished from neo-Darwinism by a substantive claim about the causes of evolution. Furthermore, the apparent incommensurability of existing internalist views with the reductionist population genetics of the New Synthesis would not reflect anything other than the fact that population genetics theory is focused almost exclusively on the consequences of sorting in mixed populations, while the consequences of bias in the introduction of variation remains an unexplored topic.

A research program based on this interpretation might have much in common with the current "evo-devo" approach. However, rather than describe these similarities, the much more suggestive differences may be outlined briefly, as follows. First, a research program focused on bias in the introduction of variation would draw from, and contribute to, the theoretical population genetics of bias in the introduction of variation. Second, this approach would have a strong focus on experimental characterization of the spectrum of spontaneous variation, as well as the molecular and developmental causes of any observed biases. Also important would be studies patterned after Alberch and Gale (1985), based on (1) characterizing the spectrum of spontaneous variation for traits of interest (either directly, or using environmental perturbation in the manner of Alberch and Gale); (2) inferring the evolutionary changes in these traits; and (3) evaluating the hypothesis that propensities of variation have influenced their evolution. Third, the experimental component of this research program would also emphasize evolution in a laboratory setting. Whereas early studies of evolution in laboratory settings tended to rely mainly or exclusively on preexisting variation (i.e., "evolution" in the sense of "shifting allele frequencies"), the introduction of novelty by mutation is a crucial process in contemporary microbial evolution experiments (Bull et al. 1997; Wichman et al. 1999).

Finally, a research program organized around the concept of bias in the introduction of variation would be both synthetic and general. Diverse biological disciplines would contribute to understanding (1) the causes of bias in the introduction of variation; (2) the theoretically expected consequences of such biases; and (3) the testable contribution of known biases to homoplasy, parallelism and directionality. The concept of an inequality in rates of variation, a measurable quantity that is universally applicable (i.e., applying to evolution in any biological or nonbiological context), would play the same unifying role as does the concept of a fitness difference (i.e., a selection coefficienct) in the neo-Darwinian view.

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