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Opinion

Disentangling variational bias: the roles of development, mutation, and selection

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The extraordinary diversity and adaptive fit of organisms to their environment depends fundamentally on the availability of variation. While most population genetic frameworks assume that random mutations produce isotropic phenotypic variation, the distribution of variation available to natural selection is more restricted, as the distribution of phenotypic variation is affected by a range of factors in developmental systems. Here, we revisit the concept of developmental bias – the observation that the generation of phenotypic variation is biased due to the structure, character, composition, or dynamics of the developmental system – and argue that a more rigorous investigation into the role of developmental bias in the genotype-to-phenotype map will produce fundamental insights into evolutionary processes, with potentially important consequences on the relation between micro- and macro-evolution. We discuss the hierarchical relationships between different types of variational biases, including mutation bias and developmental bias, and their roles in shaping the realized phenotypic space. Furthermore, we highlight the challenges in studying variational bias and propose potential approaches to identify developmental bias using modern tools.

Highlights

Population genetics often assumes that random mutations lead to a uniform distribution of phenotypic variation, which is then acted upon by natural selection.

However, growing evidence suggests that the phenotypic variation available to natural selection is not isotropic, but rather constrained and biased by various factors, including genetic architecture, mutation, and developmental systems. Such limitations and biases in the variability of phenotypic characters are referred to as variational bias.

It is critical to clarify what we measure when we measure variational bias, and how different types of variational bias relate to one another.

A more rigorous investigation into the role of developmental bias in the genotype-to-phenotype map is needed to bridge micro-evolution and macro-evolution.

What is variational bias

The observed diversity of life on Earth represents a fraction of theoretically possible phenotypes in living organisms [1–4]. Indeed, the fossil records demonstrate prolonged periods of stasis in numerous lineages, and convergent evolution is widespread [5,6]. Yet, an enduring fundamental assumption of modern evolutionary theory is that 'universal variability — small in amount but in every direction' is a key factor governing the agency of natural selection [7,8]. For example, Fisher's geometric model assumes that mutations are isotropic (i.e., the magnitude and direction of effects prior to selection are uniform with respect to the phenotypic space) [9–11]. This assumption remains a dominant paradigm in population genetics, despite mounting evidence challenging its universality [12,13]. Phenotypic variation is profoundly structured, and variation and evolution are related at multiple timescales [14–16]. While functional and population geneticists have made great progress towards an understanding of heredity, mapping genotype onto phenotype, and the mechanisms and consequences of natural selection, the structure of variation accessible to selection remains elusive [17]. The structure of variability leads to variational constraints, which are limitations and biases in the variability of phenotypic characters. These constraints represent one of the central classes of evolutionary constraint [18]. These constraints are the outcome of a broader set of factors, including genetic architecture, mutation, and development, and are observed at all levels of biological organization (Figure 1, Key figure). Different types of constraints can affect evolutionary processes – and phenotypic outcomes – in distinct ways. It is thus critical to clarify what we measure when we measure variational constraints, and how different types of variational bias relate to one another, as they are frequently intertwined.

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Key figure

Hierarchical relationships of the concepts and empirical measurements related to variational biases

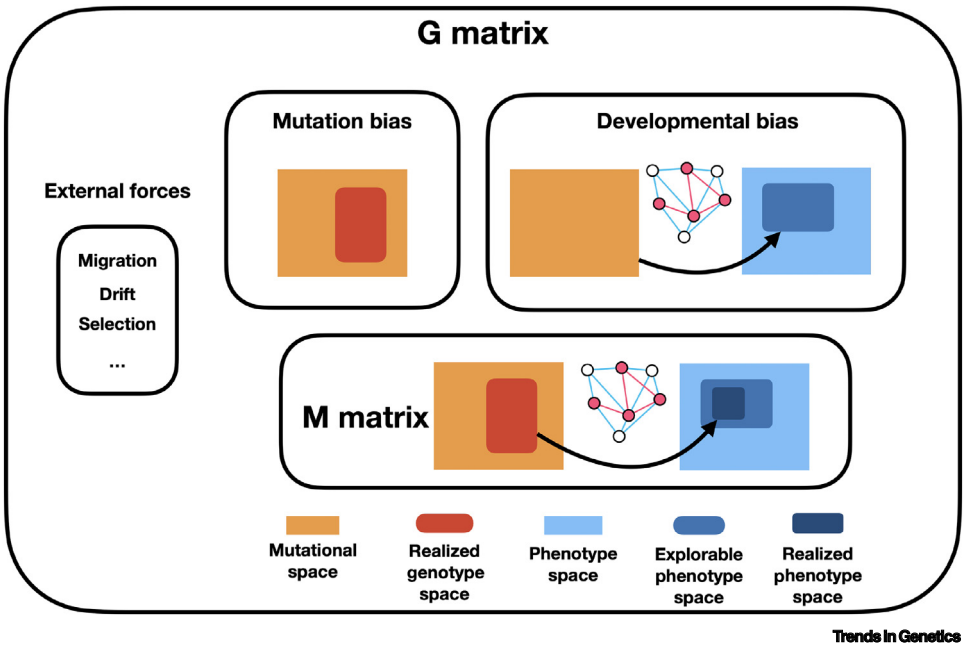

Figure 1. Because of mutational bias, a subset of genotype is more likely to occur in the genotype space of possible mutation. The genotype space is translated to the phenotype space through the process of development, which can impose developmental bias. Not all phenotypes in the phenotype space are accessible due to developmental bias, which leads to an explorable phenotype space that is a subset of the total possible phenotype space. Mutation accumulation (MA) lines capture both the mutation bias and developmental bias, which leads to a realized phenotypic space that is a subset of (explorable) phenotypic space. Therefore, the 'explorable' phenotypic space is influenced solely by developmental bias while the 'realized' phenotypic space is a result of both mutation and developmental bias as captured by MA lines. Other evolutionary forces such as selection, migration, and drift interact with mutational variation (**M**) in the realized phenotype space to shape **G**.

Identifying the sources of variational bias

Variational constraints are routinely characterized by measuring the linear association between traits in a population, and covariance matrices derived from these phenotypic measurements will capture different aspects related to the causes and consequences of variational bias [15,19–24]. For example, genetic information summarized by the additive genetic variance–covariance matrix – commonly referred to as **G** – is a common measure of variational constraint in plant and animal breeding. **G** specifically describes trait covariance due to pleiotropic alleles, wherein variation at a single locus has effects on multiple traits, or due to linkage disequilibrium of two loci that are strongly associated in populations [22,25–27]. Although the role of linkage disequilibrium may be negligible, it is not straightforward to distinguish linkage disequilibrium from pleiotropy empirically in generating **G** [28–31]. In evolutionary quantitative genetics, **G** represents an important constraint because it describes the degree to which the genetic architecture (i.e., how traits are genetically connected to each other) may determine the response of a population to selection [32,33]. Indeed, the genetic (co)variance is the most relevant parameter to the concept of 'evolvability', viewed as the population's capacity to

respond to directional selection [34,35]. The context-dependency of **G**, the evolution of evolvability itself, and how evolvability predicts the trait divergence and stasis under selection has garnered much of the empirical and theoretical attention to variational bias over recent decades [16,19,36–52].

While **G** is a measure of the amount and structure of standing genetic variation, new variation generated by new mutations can be characterized by a mutational variance–covariance matrix **M** [15,53]. The **M**-matrix can be estimated through mutation accumulation (MA) experiments under a relatively selective-neutral environment and small effective population size. While **M** itself provides information about the genotype-to-phenotype map, and hence developmental bias, it also captures bias caused by heterogeneous mutation rates and mutation spectra across the genome (Figure 1) [54–61]. In other words, **M** can reflect the inherent limitations in the genotype space that favor specific mutational outcomes (e.g., more mutable single nucleotide, transition-transversion bias). Thus, **M**, acquired through MA experiments, includes developmental bias, but is not limited to developmental bias [55]. Mutation bias is the bias specifically produced during the mutational process (Figure 2 left)without including the effects of development on phenotypes (Figure 2 middle). Thus, **M**, the mutation variance–covariance matrix, naturally captures both developmental and mutation bias.

The magnitude and direction of **G** can provide information about the degree to which evolutionary constraints may be present in a population. **G** is the product of many factors, including development, mutation, selection, drift, migration, and inbreeding [29,62–67]. The specific mechanisms and relative contributions of various factors in shaping and maintaining **G** remain poorly understood. A major complication is that, almost certainly, these contributions can dramatically differ between different sets of traits or trait combinations. Empirically, the contributions of **M** and

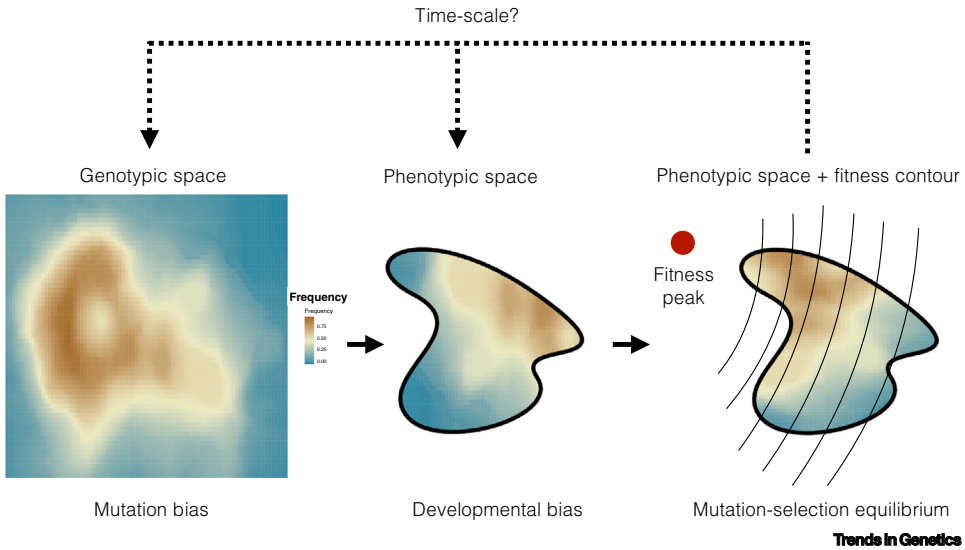

Figure 2. A hypothetical scenario showing influences of mutation bias and developmental bias on adaptation. Mutations are biased such that some mutations are more likely to occur than others (left). Developmental processes then translate genotypic variation into phenotypic space, potentially imposing developmental bias (middle). Such biased phenotypic distribution interacts with drift and selection to form the distribution of population under mutation-selection equilibrium (right). However, both mutation bias and developmental bias can evolve in response to selection. Whether the timescale of the evolution of developmental and mutation bias is longer than the trait adaptation is often unknown. Unbroken arrows represent processes occurring over short timescales, while broken arrows indicate processes that possibly occur over similar or longer evolutionary timescales.

selection to shaping **G** can be inferred by comparing **G** to **M**, or comparing **G** to γ, the matrix describing multivariate nonlinear selection. If **G** is shaped by **M**, then in equilibrium, **G** should be proportional to **M** [68]. A recent simultaneous estimate of both **G** and **M** in the same *Drosophila serrata* population indeed shows some proportionality between the two matrices, showing a contribution of mutation bias to additive genetic variation [53]. Surprisingly, in this population, **M** appears to be more constrained than **G**, which implies that selection can act to break the constraints imposed by mutation, contrary to the usual assumption that stabilizing selection increases genetic correlations. This result illustrates how nonintuitive and case dependent the shaping of **G** might be. Furthermore, other multivariate analyses suggest differences between **G** and **M** [69–74]. Notably, Houle and colleagues found **G** and **M** to be markedly similar for wing traits in *Drosophila melanogaster* and **M** reliably predicts patterns of wing divergence across drosophilids [15], although how well they align is subject to debate [75,76].

One distinction between **G** and other variational constraint is that developmental and mutational biases can vary among individuals and genotypes both empirically and in theory [74,77–80], while **G**, or more broadly phenotypic correlation, is a measure of a given population. The (co)-variance in **G** explained by each polymorphic locus is affected by allele frequencies and the magnitude of allelic effects in an individual [81,82].

Theoretical and empirical studies suggest that **M**-induced genetic correlations tend to be more stable than genetic correlation caused by selection, implying that identifying the mechanisms causing genetic correlations may help us understand the evolution of **G** [29,83]. Furthermore, the genetic architecture of **G** may be informative in inferring the drivers of **G** (selection versus **M**), since those structures of linkage disequilibrium and pleiotropy in the genome are footprints of distinct forces when inducing and maintaining **G**. Selection can also reshape **M** (Figure 2), although the timescale on which the evolution of **M** occurs – relative to phenotypic evolution – is unclear; we may be able to treat **M** as constant under most evolutionary scenarios [74,80].

Collectively, we argue that clear and distinctive definitions of variational biases at different levels are needed to ensure effective communications and nuanced analyses in the future (Figure 1).

Developmental bias in the production of phenotype

An important aspect of genotype-to-phenotype maps is that they are highly nonlinear and structured in nature. Therefore, random mutations do not necessarily produce random phenotypic changes. The distribution of phenotypic variants arising from genetic and environmental variation is influenced by the developmental processes that transform the embryonic phenotype into the adult form [84,85]. This developmental process biases the generation of phenotypic variation. This bias stems from the structure, character, composition, or dynamics of development, contrasting with the assumption of isotropic variation, and ultimately results in developmental bias [2,78,86–88].

Numerous empirical studies have hypothesized that an organism's developmental system shapes the trait–trait (co)variance observed in **G**, which has been described as phenotypic integration [18]. Development is therefore a critical factor in shaping the variational bias reflected in **M** and **G** [89]. Substantial evidence supports that this developmental bias is common [55,90–93]. For example, the developmental regulation of tetrapod limbs generates biases in the number and distribution of digits and limbs [94,95]. Interactions among components in a developmental system bias trait–trait relationships, as seen in insects and pigment coloration of insect wings [96,97].

While selection ultimately shapes developmental systems, such intrinsic biases from developmental systems are likely to be an important component of phenotypic evolution [78,87,92,98–100]. Firstly, the bias in genotype and phenotype production stands as a phenomenon distinct from phenotypic adaptation, with each being subject to separate evolutionary dynamics [36,98]. Conventionally, it is thought that developmental biases evolve more gradually than the traits that they influence [36]. Secondly, although certain parts of developmental systems remain evolvable and susceptible to selective pressures, prevailing global constraints resist alteration [86,101]. Exemplifying this notion, resource acquisition is limited due to chemical, thermodynamic, and mechanistic constraints [102–104], leading to trade-offs between, for example, growth rate and yield in *Escherichia coli* [102], or between spore number and quality in *Dictyostelium discoideum* [105]. Another well-known example comes from the metabolic scaling law, which states that metabolic rate scales with body mass to the power of 3/4. A wide range of organisms over several orders of magnitude in body mass conform to this law. Of course, one could certainly argue that it is purely natural selection that drives these points to fall along a predictable trajectory throughout evolutionary history. But there are theoretical arguments that demonstrate such metabolic scaling is caused, at least in part, by physical forces imposing constraints, or by biases in patterns of energy allocation [106–108].

Importantly, the relationship between adaptation of traits and developmental bias is not simply one of opposition, but is instead the result of a continuous dynamic interaction. Therefore, we can only ask whether developmental bias alters evolutionary trajectories in relatively short timescales [18]. For instance, two regulatory networks may yield similar functional outputs but differ in their variational properties, leading to different evolutionary biases [109]. However, over a timescale of macro-evolution, it is difficult to disentangle the contribution of developmental bias and selection in phenotypic adaptation. This difficulty arises because, first, the bias in phenotypic production itself can evolve. Second, reconstructing the evolutionary history of variational bias is not straightforward unless the mutation and developmental bias remain relatively constant over macro-evolutionary timescales. An outstanding practical problem is: can we consider developmental bias as constant and, if so, over what timescales? (Figure 2).

Emerging methods for measuring developmental bias

The evolutionary significance of developmental bias has long been controversial because, as we argue, it can be difficult to accurately diagnose. Natural selection and random genetic drift strongly affect the patterns of phenotypic variation within and between populations, making it unsatisfying to rely solely on measurement of existing phenotypic variation when attempting to identify developmental bias [26,110]. Given that both developmental bias and selection could create similar phenotypic distribution in natural populations, it is generally difficult to distinguish between the two [18,32]. To quantitatively investigate developmental bias, researchers need to assess the propensity of phenotypic production prior to selection rather than merely observing the current state of variation [98].

One traditional approach to distinguish between the effects of developmental bias and selection is through MA lines to assess the spectrum of phenotypic variation generated by *de novo* mutation in the absence of selection [15,79,111]. However, as mentioned earlier, *de novo* mutation captures not only the propensity of the developmental system to vary, but also heterogeneity in mutational rates and spectra across a genome, which constrains the mutation in genotype space (Figure 1, e.g., more mutable single nucleotide, transition-transversion bias, etc. [54–56,60,112]). Alternatively, certain well-characterized developmental systems, such as mammalian tooth morphology [113] and vulva development [114], can be sufficiently well modeled so that a large number of perturbations can be simulated to evaluate the variability *in silico*. However,

this method is feasible only for a few systems for which we have relatively complete knowledge of intricate developmental dynamics.

Another approach to diagnose developmental bias is to measure the symmetry of the left and right sides of the same organism – so-called 'fluctuating asymmetry' – [55,115,116], which share both a genome and environment. However, the developmental process can introduce asymmetry in morphological traits due to inevitable consequences of molecular stochasticity, often interpreted as developmental variability. The developmental variability is specifically quantified as the variance–covariance matrix of deviation from symmetry, which describes the correlated shape changes due to the noise. A recent study showed that developmental bias quantified using such internal variability in the dipteran wing predicts its evolution on both short and long evolutionary timescales [55]. Ultimately, addressing the evolutionary role of developmental bias requires studies in more systems. We thus propose alternative and existing methods to characterize developmental bias, thereby reducing reliance on the limited developmental systems exploited in past studies.

In line with the concept of fluctuating asymmetry, there are multiple ways to assess the propensity of the system to vary by inducing mild and random (environmental or genetic) perturbations. The phenotypic variation in a genetically identical population, under the same environmental conditions, has often been referred to as intra-genotypic variability [117,118] or phenotypic variability [119–121], which is thought to be an emergent by-product of developmental processes [121]. Such variability reflects both the results of stochasticity in molecular interactions and of external sources caused by microenvironmental variation [122–124] and has been explored theoretically within evolutionary contexts [125–128]. These extrinsic and intrinsic small random fluctuations interact with developmental systems and give rise to the phenotypic variability. Thus, we hypothesize that the variational properties induced by random small perturbations most likely reflect the inherent attributes of the system rather than the certain direction of the perturbation (e.g., changes of a nutrient level, single gene knockout etc.). Indeed, such phenotypic variability has been used to characterize developmental systems in many studies [129–131], though these studies have not explicitly addressed developmental bias. For example, examining variational properties under identical environmental conditions across clonal cells helps quantify the developmentally correlated traits in yeast morphology [131].

Over the past few decades, studies of gene expression evolution have proliferated owing to the reduced cost of RNA sequencing. This has led to a surge of studies on canalization and modularity and phenotypic integration at the molecular level has followed [132–135]. These concepts are all connected to the classic notion of developmental constraint [14]. Yet, few studies have used expression variability to address these questions. Much efforts in gene expression variability have been taken to assess the genomic, epigenetic, and topological features in determining the gene-specific expression variability level [124,136]. We argue that genome-scale expression variability data can be exploited to investigate the bias in the production of gene expression and, ultimately, contribute to our understandings of evolution in gene expression [137].

Another way to impose random perturbations is through random mutation. As discussed earlier, the variants captured in MA experiments account for the heterogeneity of mutation rates and other mutational bias across the genome (Figure 1), which is an informative way to unravel the role of mutation during evolution. Unfortunately, MA studies provide a very limited view of the distribution of mutational effects because the number of spontaneous mutations sampled in each study tends to be very low [138]. One approach to ameliorate these limitations is to

introduce mutation without incurring mutational bias during *de novo* mutation. For example, genome-wide mutagenesis [138], as opposed to *de novo* mutation, provides an empirical investigation of bias in phenotypic production and reveals a greater neutral expression divergence than commonly used models of phenotypic evolution. Examples of mutagenic populations include *Arabidopsis* T-DNA insertion lines [139], yeast single-cell deletions [140], and, more recently, single-cell technologies such as Perturb-Seq [141] and CRISPR-mediated genetic screening [142]. A similar outcome and mutational landscape of a given trait (trait combination) from a wide-range of variants across the genome would be indicative of developmental or mutation bias. Alternatively, artificial recombinant populations provide mutational perturbative materials to examine the propensity of the system to vary [29,143].

Recent advances in high-throughput phenotyping facilitate the goal to disentangle variational bias. These advances include both large-scale platforms for growing and manipulating both prokaryotic [144] and eukaryotic [145] organisms, but also imaging and other systems for measuring traits at high resolution [146]. One advantage of automated, often time-sequenced phenotyping of highly replicated experimental populations is the possibility of reducing measurement error, thus increasing the power to detect subtle phenotypic differences among clonal, inbred, or recombinant populations.

Concluding remarks

One long-standing proposition for bridging the mechanistic gap between micro- and macro-evolution has been through the study of evolutionary developmental biology and ontogeny [90,147]. Here, we argue that progress in integrating micro- and macro-evolutionary theory has been hampered by the common assumption in population genetics that genotype-to-phenotype mapping is a straightforward exercise emerging from an invariant distribution of mutational effects. There is substantial evidence that variational bias is common. Such bias may stem from factors in mutation, genetics, and development. We argue that clear and distinct definitions of and diagnostic criteria for variational biases at different levels of organization (Figures 1 and 2) are needed to help better understand the role of variational bias in adaptation and how evolution shapes it. Furthermore, as we have shown, developmental bias is notoriously difficult to establish empirically. We thus review and propose approaches aimed at identifying developmental bias and testing for its role in shaping phenotypic evolution (Table 1). In particular, we argue that a large number of untargeted and random perturbations can be exploited to assess the propensity of the system to vary and, hence, the bias of phenotypic production. Collectively, we present challenges in studying variational bias and its role in shaping evolutionary history and impacting future adaptation (see Outstanding questions).

Outstanding questions

How does the indirect adaptation of variation generation (mutation and developmental bias) to environments contrast with the direct adaptation of phenotypic traits?

Does the rate of adaptation differ among different types of variational bias?

Is developmental bias a relatively more stable component than genetic variance–covariance structures?

How do we utilize genome-scale expression variability data to investigate the bias in the production of gene expression and contribute to our understanding of gene expression evolution?

How do we characterize developmental bias without depending on specific developmental systems?

Can we consider developmental bias as constant and, if so, at what timescales?

How does developmental bias shape macro-evolutionary patterns?

Table 1. Summary of potential empirical methods to detect developmental bias

Type	Source of perturbations	Caveats
Mutation accumulation experiment (MA)	*De novo* mutation	Includes both mutation and developmental bias; the sample size is limited
Untargeted mutagenesis (e.g., chemical mutagen ethyl methanesulfonate (EMS) [138], T-DNA [139])	Induced mutation	Possible bias from large effect size mutations
Recombinant strains	Recombinant mutation	Possibly involve selection, since alleles in the parents are fixed
Phenotypic variability using clonal individuals	Micro-environmental variation	Potential nonnegligible effect of measurement error
Perturb-seq [141] (also known as CRISP-seq and CROP-seq)	Induced mutation	Suitable for single-cell transcriptomic studies

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Declaration of interests

The authors have no conflicts of interest to declare.

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