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OVERVIEW

Systems biology of robustness and homeostatic mechanisms

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All organisms are subject to large amounts of genetic and environmental variation and have evolved mechanisms that allow them to function well in spite of these challenges. This property is generally referred to as robustness. We start with the premise that phenotypes arise from dynamical systems and are therefore system properties. Phenotypes occur at all levels of the biological organizational hierarchy, from gene products, to biochemical pathways, to cells, tissues, organs, appendages, and whole bodies. Phenotypes at all these levels are subject to environmental and genetic challenges against which their form and function need to be protected. The mechanisms that can produce robustness are diverse and several different kinds often operate simultaneously. We focus, in particular, on homeostatic mechanisms that dynamically maintain form and function against varying environmental and genetic factors. Understanding how homeostatic mechanisms operate, how they reach their set point, and the nature of the set point pose difficult challenges. In developmental systems, homeostatic mechanisms make the progression of morphogenesis relatively insensitive to genetic and environmental variation so that the outcomes vary little, even in the presence of severe mutational and environmental stress. Accordingly, developmental systems give the appearance of being goal-oriented, but how the target phenotype is encoded is not known. We discuss why and how individual variation poses challenges for mathematical modeling of biological systems, and conclude with an explanation of how system population models are a useful method for incorporating individual variation into deterministic ordinary differential equation (ODE) models.

This article is categorized under:
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1 | INTRODUCTION

Bacteria, yeast, animals, and plants are dynamically changing systems that operate in complex and variable environments. They are composed of diverse physiological, biochemical, and genetic systems that provide a division of labor and compartmentation of various specialized functions. All these functions need to be integrated to respond to daily and seasonally changing variables in the environment, such as nutrition, temperature, water availability, toxins in food (e.g., plant secondary compounds), and pathogens that populations experience throughout their existence. Phenotypes are emergent properties of

biological systems that arise from multivariate nonlinear interactions among many genetic and environmental components. Moreover, phenotypes are not steady-state properties but are dynamically maintained in the face of continually changing internal and external variables.

All organisms are subject to large amounts of genetic and environmental variation and have evolved mechanisms that allow them to function well in spite of these challenges. The ability to resist otherwise deleterious genetic or environmental variation goes by the name "robustness" (Wagner, 2005). Robustness is an observed property of a system but does not imply a particular mechanism. Indeed, robustness and its complement, phenotypic plasticity, are often studied as a statistical properties of phenotypes that can be measured, quantified, and compared in organisms in different environments or with different genetic backgrounds (Badyaev, 2005; de Visser et al., 2003; DeWitt & Scheiner, 2004; Via & Lande, 1985). Studies that try to elucidate the actual mechanisms underlying robustness are less common, although there is a substantial body of theoretical work that examines the kinds of mechanisms that can, in principle, buffer the effects of either genetic or environmental variation (Masel & Siegal, 2009; Felix & Barkoulas, 2015; Felix & Wagner, 2006; Lehner, 2010; Meir, von Dassow, Munro, & Odell, 2002; Nijhout & Reed, 2014; Stewart, Parsons, & Plotkin, 2012; von Dassow, Meir, Munro, & Odell, 2000; Wagner, 2008; Whitacre & Bender, 2010).

Phenotypic stability is maintained by myriad overlapping homeostatic mechanisms at the genetic, cellular, biochemical, tissue, and whole-organism levels that ensure that phenotypes are robust to genetic and environmental variation. By genetic variation, we mean not only the presence of mutations but also that even when the genes are identical their expression levels vary on average by 25% between individuals (Boeuf, Keijer, Franssen-Van Hal, & Klaus, 2002; Oleksiak, Churchill, & Crawford, 2002). On the other hand, animals, plants, and bacteria also often have plastic responses to environmental variables, which means that many different phenotypes can correspond to a single genotype. Since natural selection acts on phenotypes, this raises the question of how selection can act on the genome if genotypes are decoupled from phenotypes by robustness and plasticity mechanisms. In fact, the ubiquitous occurrence of robustness and plasticity make the concept of a unique genotype–phenotype mapping seem facile and misleading.

Biological systems are extremely diverse and complex, and each is embued with homeostatic mechanisms, adaptive mechanisms, and enormous variation in the component parts. Thus, understanding the mechanisms by which they work has been and will continue to be very challenging. It is this understanding that is key to making reliable predictions about the evolution of biological systems in a changing world, and about the likely consequences of interventions.

In this review we focus on mechanisms, both theoretical and empirical, that make traits and organisms robust. We start with the premise that phenotypes arise from dynamical systems and are therefore system properties. Phenotypes occur at all levels of the biological organizational hierarchy, from gene products, to biochemical pathways, to cells, tissues, organs, appendages, and whole bodies. Phenotypes at all these levels are subject to environmental and genetic challenges against which their form and function need to be protected. As we will see, the mechanisms that can produce robustness are diverse, and several different kinds often operate simultaneously.

Systems need to operate reliably under many diverse circumstances, but they also need to be poised to respond adaptively to a new circumstance, and change on both short and long time scales (Nijhout, Sadre-Marandi, Best, & Reed, 2017; Padilla & Tsukimura, 2014). So robustness needs to be balanced with plasticity. On a short time scale, as we will see below, some variables change a lot so that other variables (concentrations and fluxes) can remain robust. This is the essence of a homeostatic mechanism: some things change so that others do not. On the time scale of the life of an organism, epigenetic mechanisms such as DNA methylation modify the genome so that the organism can adapt to changing circumstances. Finally, on the time scale of evolution, populations acquire new distributions of genotypes to cope with varying and changing circumstances.

Biological systems are seldom if ever at steady-state. Indeed, they are pushed away from their steady state by continual changes in input and demand. In a trivial but universal case, the steady-state in the absence of nutrient input is death. With a constant nutrient input the system can be maintained at a different steady-state that will depend on the amount of nutrient and the internal properties of the system. Nutrient input is, however, never constant: it fluctuates hourly, daily and seasonally. Yet some of the nutrient-dependent properties (such as ATP production, body temperature, and glucose concentration) do not fluctuate with varying nutrient input. They remain quite stable over a broad range of time scales. This stability is due to the fact that other components of the system change continually in order to maintain stability of these critical phenotypes. Robustness that is maintained through such dynamic processes is generally referred to as homeostasis. In homeostasis, some structures or processes are kept dynamically stable whereas other processes adjust, as needed, to stabilize them. Although homeostasis is best known from physiological systems, it also occurs in developmental systems, cellular systems and biochemical systems.

2 | THE PROBLEM OF VARIATION

No two individuals are genetically alike, nor do they experience the identical environmental variation. The 1000 Genomes Project (The 1000 Genomes Project C, 2015) found that a typical human genome contained 10,000–12,000 sites with peptide-

sequence-altering variants and some 2,000 variants associated with variation in complex traits. Mutational variation aside, there is also a great deal of individual variation in gene expression and protein expression levels. Expression of genes can vary by a factor of two or more (Oleksiak et al., 2002; Whitney et al., 2003) and protein expression levels vary 1.5 to twofold (Ahmed et al., 2013). The coefficient of variation (CV) of gene expression in mice is 0.2–0.4 (Holmes et al., 2017), and Miller, Galecki, and Shmookler-Reis (2001) reported that in humans 80% of the genes tested had CVs >0.3 and that 56% had CVs > 0.5. In humans, protein level variation has a CV of 0.1–0.3 (Sigal et al., 2006). Some of this individual variability is transcriptional noise (Ramsey et al., 2006), although there is some correlated variation (Holmes et al., 2017; Whitney et al., 2003), perhaps due to variation in shared regulatory processes, suggesting that the variability may also be functional.

Metabolic and biochemical systems are also subject to both genetic and environmental variation. Hourly, daily, and seasonal variation in nutrient quantity and quality, physical activity, reproduction, temperature, and weather, all affect the rates of biochemical processes.

Variation in gene and protein expression will change the flux through biochemical pathways and the accumulation, depletion, and redistribution of metabolites. The concentrations of metabolites and biochemical species exhibit a great degree of individual variability. Data from the National Health and Nutrition Examination Survey (NHANES) database (https://www.cdc.gov/nchs/nhanes/Default.aspx) show that most blood metabolites have a more than twofold variation around the mean (<0.5× mean to >2× mean). Chaleckis, Murakami, Takada, Kondoh, and Yanagida (2016) report that CVs of metabolites and other compounds in human blood range from 0.1 to 2.5, and data from Saito et al. (2016) likewise show a great range of individual variation of blood compounds with an average CV of 0.41 (Figure 1). Chaleckis et al. (2016) suggest that compounds with a very low CV are tightly controlled and their variation outside those bounds is therefore likely to be associated with disease. Epidemiological studies of cancer and other diseases have shown varying degrees of correlation between the incidence of disease and a diversity of environmental, biochemical, and genetic factors (Curtin et al., 2004; Levine et al., 2010; Ulrich et al., 2005; Ulrich et al., 2008).

One important advantage of variation is that some segments of a population of cells or organisms can more easily respond to changing circumstances. Nevertheless, the evident enormous biological variability poses serious questions for a mechanistic understanding of how biological systems work. How is it that members of a species are incredibly different locally in space and time but globally are pretty much the same? How does that happen? And, if there's so much variation, what does it mean biologically to make a deterministic mathematical model with fixed coefficients? We consider both of these questions below.

3 | HOMEOSTASIS

A large diversity of stabilizing mechanisms have evolved to maintain biologically important functions within a reasonably narrow range in the face of variation in genetic and environmental inputs. The best known of these are the physiological homeostatic mechanisms that maintain a stable body temperature in endotherms, and those that maintain a stable ionic composition of blood, blood pH, blood glucose, blood osmolarity, blood volume, and blood pressure (Guyton, 1981). These homeostatic mechanisms are complex and require an integration of functions distributed among organs and cells throughout the body.

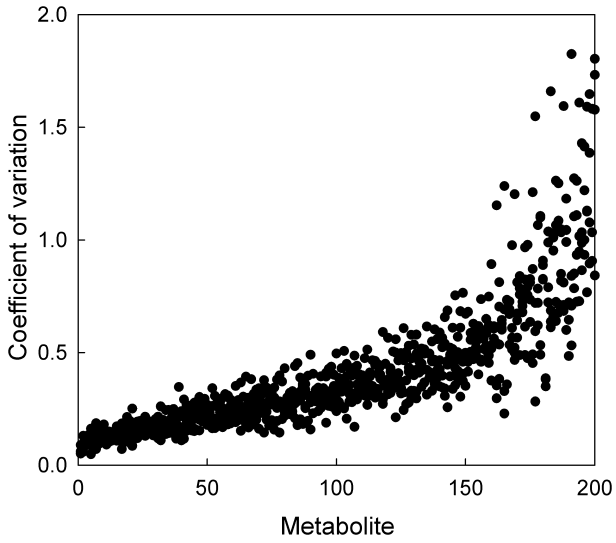

FIGURE 1 Coefficients of variation of 200 metabolites from 60 individuals (B.sed on data from Saito et al., 2016). Values range from 0.02 to 1, with a mean of 0.41. If a population has a CV of 0.41 and a mean of μ, this means that 32% of a population will have values either>1.41×μ and < 0.59×μ

Homeostatic stabilizing mechanisms are not restricted to physiological systems. They also occur in biochemical and metabolic systems, as well as in cellular and developmental systems. In biochemical and metabolic systems, homeostatic mechanisms ensure stability of critical functions in the face of hourly, daily, and seasonal variation in inputs and demands. In cellular systems, homeostatic mechanisms ensure stability and integrity in tissues with rapid cellular turnover. In developmental systems, homeostatic mechanisms make the progression of morphogenesis relatively insensitive to genetic and environmental variation so that the outcomes vary little even in the presence of severe mutational and environmental stress. The operation of such developmental regulatory mechanisms is most evident in cases of regeneration, where missing parts are reconstructed to their original form starting from very different initial conditions, but is also evident throughout ontogeny and gives developmental systems the appearance of canalization (Waddington, 1942; Waddington, 1956; Waddington, 1957), self-organization and goal-orientation, which has led to the unfortunate metaphor of the "developmental program" encoded, somehow, in the genome (Nijhout, 1990).

4 | PHENOTYPES ARE DYNAMICAL SYSTEMS

Biological structures and functions require continual maintenance. Entropy tends to degrade highly ordered systems, and the stability of a biological system depends on processes that counteract the relentless march toward disorder. Even apparently stable features, like the dead exoskeleton of an arthropod, is continually maintained by epidermal cells and their pore canals (Wigglesworth, 1965). Similarly, vertebrate bones are continually renewed, recycled, and remodeled by osteoblasts and osteoclasts. The epidermis and the lining of digestive systems are continually sloughed off and regenerated, and muscle mass increases and decreases with use and disuse. Phenotypes are therefore dynamical systems, and are not characterized by particular static patterns of gene expression: even within a tissue, seemingly identical cells have dramatically different patterns of gene expression (Eberwine & Kim, 2015).

5 | MECHANISMS OF HOMEOSTASIS

The operation of a homeostatic mechanism can be recognized by a chair-shaped curve that relates a particular function or output to an input or parameter (Figure 2a). At very low and very high input levels the output is proportional to the input, but in the intermediate range of inputs the output is almost constant. The input could be an environmental variable such as temperature, a macro- or micro-nutrient, or an environmental chemical or toxin. The output function could be a metabolic flux, a metabolite concentration, body temperature, blood pH, blood pressure, kidney filtration rate, lipid storage, or any other physiological feature that is maintained at some constant value or is part of the mechanism that keeps critical features from fluctuating. Almost all homeostatic mechanisms operate successfully over only a moderate range of the input parameter. Outside of that range they function poorly or not at all leading to change that is proportional to changes in the input parameter. We call this phenomenon "escape from homeostasis" and have explained how it can be associated with disease processes and states (Nijhout, Best, & Reed, 2014, 2015).

A variety of mechanisms can produce chair-like curves (Antoneli, Golubitsky, & Stewart, 2017; Golubitsky & Stewart, 2016; Reed et al., 2017). In general, some kind of feedback or feedforward mechanism is required, but these regulatory interactions can be implemented in a variety of ways. Figure 2 shows several motifs that can produce homeostasis and chair-like response curves (Majumder et al., 2004). Feedback inhibition (Figure 2b) is a well-known regulatory mechanism in biochemistry and physiology. In feedback inhibition, a product inhibits an earlier synthetic reaction so as the product accumulates its synthesis is repressed. Depending on the parameter values, this motif can produce a chair-like curve that relates Z to I (Figure 2a). Homeostasis can also be provided by feedforward excitation (Figure 2c). Feedforward excitation occurs in a biochemical network when a substrate activates the enzyme that removes a product, Z. Increasing the input, I, causes X to increase, and as X gets larger both the rate of synthesis and the rate of removal of Z increase. This mechanism can stabilize Z in the presence of variation in I and can result in a chair-like curve that relates Z to I. In the parallel inhibition motif (Figure 2d), Z inhibits the production of X, and X inhibits the removal of Z. This scheme stabilizes Z against variation in its input, I, and can produce a chair-like curve that relates Z to I. If a feedback loop is slow, for instance, if it involves several intermediate steps, there will be a delay in the feedback signal, which will result in oscillations around the stable plateau (Duncan, Best, Golubitsky, Nijhout, & Reed, 2018).

These homeostatic mechanisms can occur singly or in various combinations. Figure 3 illustrates a biochemical network in which all three motifs operate. This network is composed of the folate and methionine cycles, and regulates a broad range of methylation reactions. The folate cycle contains the rate limiting steps of de-novo purine and pyrimidine synthesis (via the TS and AICART reactions), DNA methylation (via the DNMT reaction), and some 150 other methylation reactions that use S-

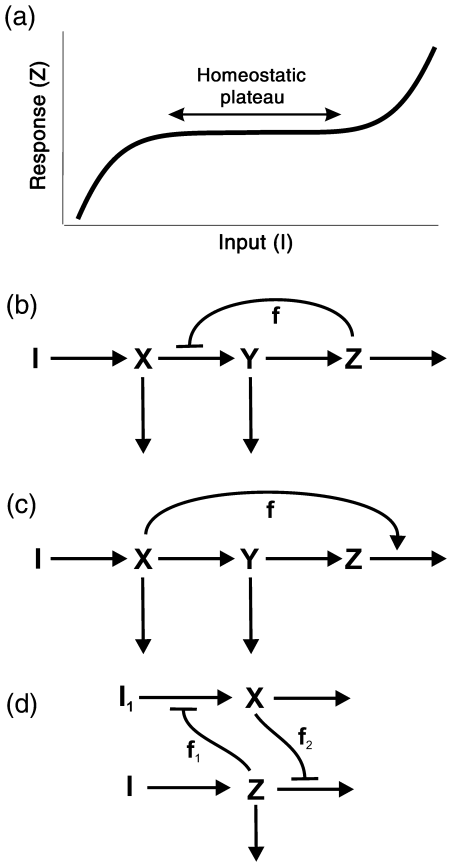

FIGURE 2 Feedback mechanisms that can produce homeostasis. (a) Chair-shaped curve characteristic of a homeostatic mechanism, with a more or less broad homeostatic plateau in which variation in an input (I in the diagrams below) has little or no effect on the output (Z in the diagrams below) of a system, flanked by regions where very low or very high levels of input produce a proportional response. Three motifs are shown that can exhibit this property, depending on parameter values and reaction kinetics. (b) A typical product feedback inhibition mechanism in which the product of a reaction inhibits an enzyme earlier in the biosynthetic chain. (c) Feedforward activation of a downstream reaction can stabilize an intermediate metabolite. (d) Mutual inhibition among parallel reactions can stabilize *Z* to variation in I. Here metabolite Z inhibits the synthesis of X, and X inhibits the breakdown of Z. (Modified from Reed, Best, Golubitsky, Stewart, and Nijhout (2017))

adenosyl methionine (SAM) as the methyl group donor (Chen & Blumenthal, 1999; Clarke & Banfield, 2001) (only 5 of these are illustrated); at any one time only a few of the possible methylation reactions are active (Chen et al., 2010; Reed et al., 2015). The black arrows in the diagram indicate biochemical reactions and the red arrows indicate regulatory interactions (arrow heads indicating activation and bar heads indicating inhibition). The overall effect of the regulatory interactions is to stabilize the rates of the methylation reactions in the face of variation in methyl group import via methionine, glycine, and serine (Nijhout et al., 2006; Nijhout, Reed, Lam, et al., 2006). Only a subset of regulatory interactions is shown in the diagram. For instance, in the folate cycle many enzymes are subject to substrate inhibition, which plays a major role in stabilizing flux through the system (Nijhout et al., 2004; Reed, Lieb, & Nijhout, 2010).

5.1 | The homeostatic "set point"

The height of the homeostatic plateau in a chair curve is the homeostatic set point. In physiological systems, set points are values to which a system returns after perturbation, or around which a system oscillates. For instance, the body temperature set point in humans is 37°C and under normal conditions it oscillates around this value with an amplitude of less than 1°C over a range of external temperatures of 13–54°C (Guyton, 1981). This stability is regulated by a complex interaction of heating, cooling, and heat transfer systems throughout the body. Many other physiological and biochemical factors have similar set points. For instance blood glucose (100 mg/dL), regulated by insulin and glucagon, blood osmolarity (285 mOsm/kg), regulated by antidiuretic hormone, aldosterone, and blood pressure sensors, pH of blood (7.4), regulated by the kidney. Significant deviations from the set point are generally pathological. The body temperature set point can be elevated to produce fever which enhances immune activity (Oleksiak et al., 2002), although too high an elevation can be fatal.

Although many of the mechanisms that bring biological variables back to their set point after deviation are well-understood, the nature of homeostatic set points is unknown. Homeostatic set points are absolute values, not relative.

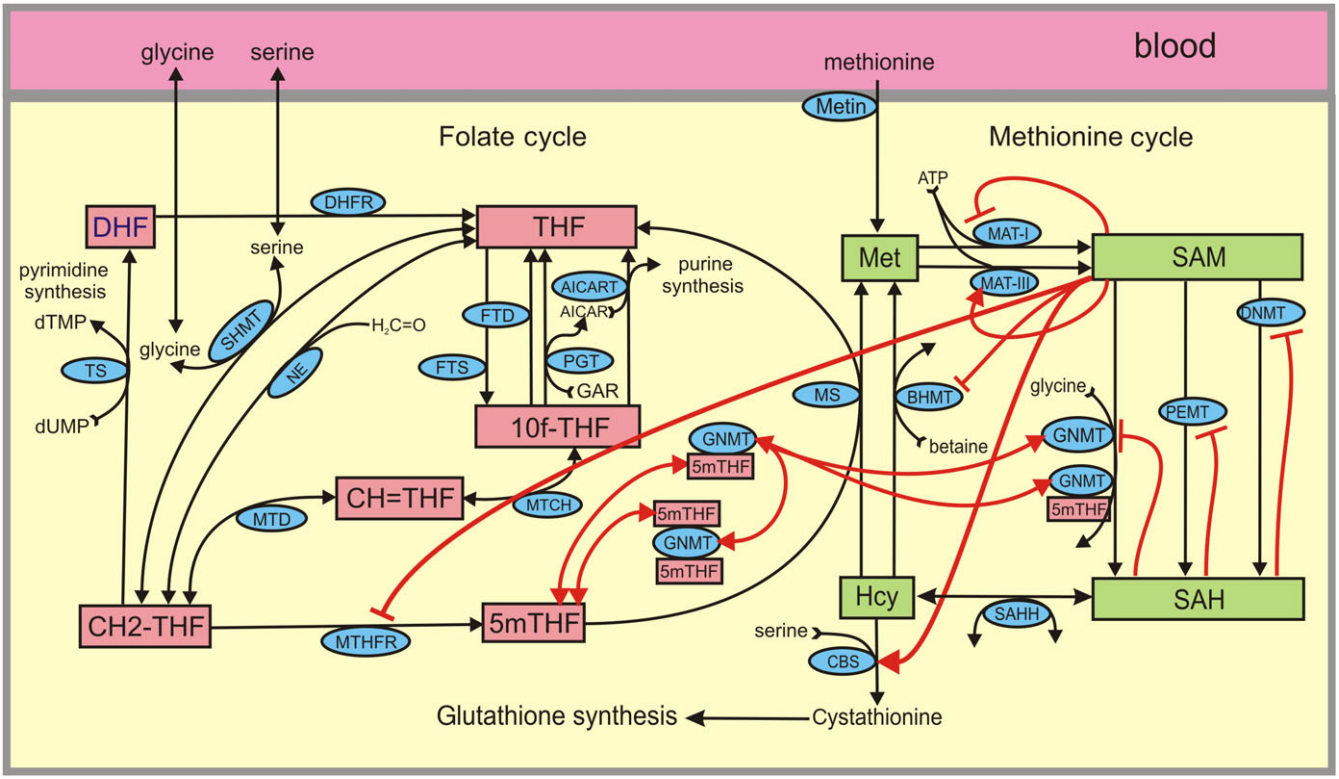

FIGURE 3 Reaction diagram for one-carbon metabolism (OCM). OCM consists of the folate cycle, the methionine cycle, and the glutathione synthesis pathway. Its function is to capture methyl groups from amino acids (methionine, serine, and glycine) that are used by a variety of methyl transferase reactions in the biosynthesis of more complex molecules. Enzymes are indicated by their acronyms in blue ellipses. Metabolites are in boxes. Black arrows indicate traditional enzymatic reactions. Red arrows indicate allosteric regulatory reactions by which metabolites alter the activities of enzymes. This metabolic network is made up of several interlocking cycles. *Enzymes*—AICART: aminoimidazole carboxamide ribonucleotide transferase; BHMT: betaine-homocysteine methyltransferase; CBS: cystathionine β-synthase; DHFR: dihydrofolate reductase; DNMT: DNA methyltransferase; FTD: 10-formyl tetrahydrofolate dehydrogenase; FTS: 10-formyl tetrahydrofolate synthase; GNMT: glycine *N*-methyltransferase; MAT-I: methionine adenosyl transferase I; MAT-III: methionine adenosyl transferase III; MS: methionine synthase; MTCH: 5,10-methenyl tetrahydrofolate cyclohydrolase; MTD: 5,10-methylenetetrahydrofolate dehydrogenase; MTHFR: 5,10-methylenetetrahydrofolate reductase; NE: non-enzymatic conversion; PGT: phosphoribosyl glycinamide transformalase; SAAH: S-adenosyl homocysteine hydrolase; SHMT: serine hydroxy methyltransferase; TS: thymidylate synthase. *Metabolites*—10f-THF: 10-formyl tetrahydrofolate; 5mTHF: 5-methyl tetrahydrofolate; CH=THF: 5-10-methenyl tetrahydrofolate; CH2-THF: 5-10-methylenetetrahydrofolate; DHF: dihydrofolate; Hcy: homocysteine; Met: methionine; SAH: S-adenosyl homocysteine; SAM: S-adenosyl methionine; THF: tetrahydrofolate. (Modified from, and absed on Nijhout et al. (2006); Nijhout, Reed, Budu, and Ulrich (2004); Reed et al. (2008); Reed, Gamble, Hall, and Nijhout (2015))

Body temperature, blood osmolarity, and pH do not depend on age, sex, race, or body mass and vary only slightly with physiological condition. The set points reside in the hypothalamus, but exactly what property sets a given set point at its particular value is unknown. This is a difficult problem to study. If the set points were variable, and systematically correlated with some other measurable variable, then some experimental manipulations might allow one to get insight into the underlying mechanism. Although we know a great deal about mechanisms that bring physiological variables back to their set points, today, none of the mechanisms that determine physiological set points are understood.

5.2 | Mechanisms of robustness

Not all robustness mechanisms have the properties of homeostatic mechanisms. For example, a reaction that is running at saturation (i.e., with a substrate concentration far above the K_m of an enzyme) is robust to variation in substrate concentration. Such reactions are typically controlled by the expression level of the enzyme. Likewise, cooperative reactions that have sigmoidal substrate-flux curves are robust to substrate variation at both low and high substrate concentrations. Such reactions can provide bistable on/off switches in response to a continuously varying input (Ferrell, 1997; Huang & Ferrell, 1996; Nijhout & Callier, 2013; Tyson, Albert, Goldbeter, Ruoff, & Sible, 2008; Verdugo, Vinod, Tyson, & Novak, 2013).

5.3 | Sequential reactions

Sequences of enzymatic reactions have a progressively more nonlinear relationship between enzyme activity and flux, with increasing chain length (Kacser & Burns, 1981) (Figure 4a). This nonlinearity can lead to robustness of flux to variation in enzyme activity: flux is no longer proportional to enzyme activity but is relatively stable over a broad range of enzyme activities (Figure 4b). Insofar as enzyme activity is proportional to the level of expression of the gene that codes for it, biochemical chains make flux through the system robust to variation in gene expression. Genetic dominance, in which a heterozygote has the same activity as one of the homozygotes, is a form of robustness to genetic variation and haplo-insufficiency, since a half dose of a gene is sufficient to drive flux, or express a phenotype, at a near normal rate (Figure 4b).

5.4 | Switch-like behavior

Many biological reactions have sigmoidal relationships between input and output (or cause and effect). Signaling cascades and phosphorylation cascades can have sigmoidal switch-like behavior with little or no response to low levels of input and a constant response at inputs above the switch-point (Ferrell, 1996, 1997) (Figure 5). For instance, the MAPK cascade, the JNK cascade and the insulin signaling network exhibit switch-like behavior of output as a function of input. In such cascades, the response becomes increasingly switch-like as one moves farther down the cascade and this may help explain why signaling cascades and hormone response networks like the insulin signaling cascade, have multiple sequential steps (Bagowski & Ferrell, 2001; Huang & Ferrell, 1996; Nijhout & Callier, 2013). The consequence is that at low inputs the system does not respond, then there is a relatively sharp transition, and at higher inputs the system responds maximally. This is an important behavior that prevents responses to small and possibly random fluctuations in input, and prevents an excessive response to a very large input.

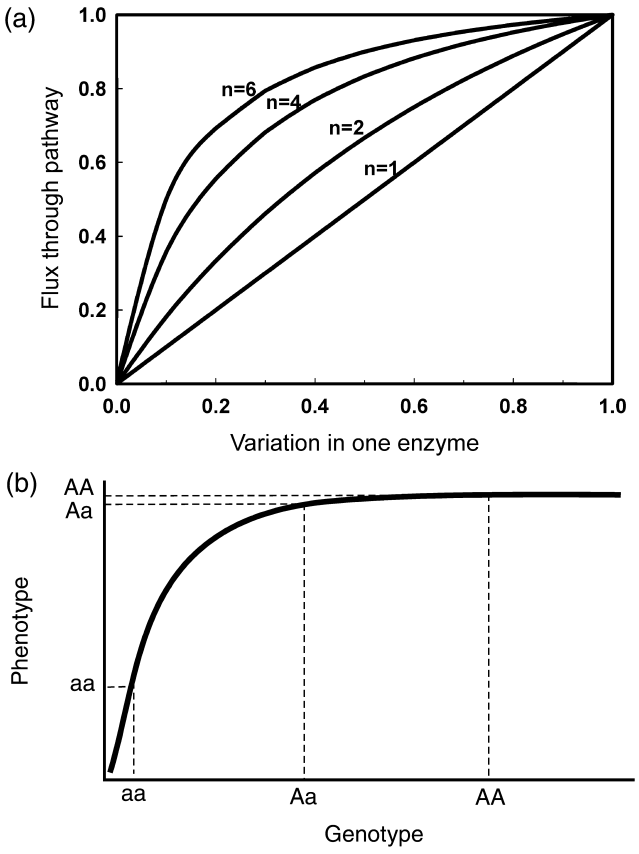

FIGURE 4 System properties of biochemical reaction chains. The relationship between flux through a sequential reaction chain and the activity of any one enzyme in the chain becomes progressively more nonlinear with increasing chain length. (a) Effect of variation in one enzyme on the flux through enzymatic chains of length n (scaled to 1). (b) Emergence of dominance from a nonlinear mechanism. A gene with two alleles (A and a) with different "activities" are shown, with alleles acting additively, so that the heterozygote (Aa) is half-way between the two homozygotes (AA and aa). The effect of the three genotypes (*x*-axis) on the phenotype (*y*-axis) is not additive, but the phenotype of the heterozygote is much closer to that of one of the homozygotes (AA). Thus the a allele is additive (co-dominant) at the genotypic level but dominant at the phenotypic level. This emergence of dominance arises in any system in which the relationship between genes and traits is nonlinear. Based on (Gilchrist and Nijhout (2001); Kacser and Burns (1981))

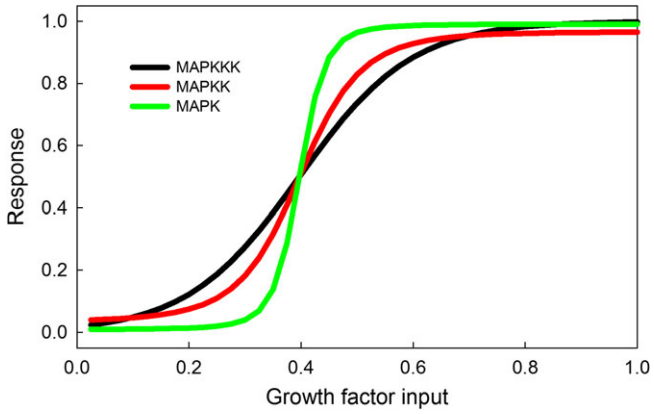

FIGURE 5 System properties of phosphorylation cascades. The mitogen activated kinase (MAPK) cascade is stimulated by a variety of growth factors and controls gene expression that leads to cell proliferation and growth. Double phosphorylations at each stage in the three-step cascade (MAPKKK - > MAPKK - > MAPK) lead to a progressively sharper sigmoidal dose–response relationships

A common mechanism for obtaining switch-like behavior between stimulus and response in a signaling cascade is to have multiple phosphorylations at each step (Ferrell, 1996; Huang & Ferrell, 1996). In the MAPK cascade a double phosphorylation of one of the steps leads to a sigmoidal stimulus–response curve with a Hill coefficient of approximately 2 (Ferrell, 1996; Ferrell, 1997). In a sequence of such reactions the Hill coefficients have an approximately multiplicative effect (Ferrell, 1996, 1997), so that at the end of the cascade the response is sharply switch-like with a high-apparent Hill coefficient. Serial reactions and multiple phosphorylation cascades are universal in metabolic, biochemical, and cell signaling networks, and the two processes just discussed (increasing nonlinearity and switch-like behavior with increasing chain length) that produce robustness to variation in gene expression and stimulus strength are therefore natural properties of those systems.

5.5 | Homeostasis and robustness in developmental systems

One of the most remarkable features of animal and plant development is its irrevocable directionality and the appearance of being goal-oriented. Severe insults during development, such a removal of part of an embryo, or of a developing appendage, can redirect development to repair the lesion and regenerate the missing parts to near normality. The ability to flexibly structure a body in early embryogenesis is called regulative development, and stands in contrast to mosaic development in which missing parts cannot be replaced (Gilbert, 1997). Later in development, the ability to reconstruct missing parts is generally referred to as regeneration.

Many authors have commented on the fact that development gives the appearance of being self-organizing and self-correcting and have suggested mechanisms by which this could occur (Kauffman, 2012; Camazine et al., 2001; Isaeva, 2012). The most common mechanism invoked for self-organizing pattern formation in development is Turing's reaction diffusion system (Turing, 1952). Turing showed that stable chemical concentration patterns can form in an initially homogeneous, but randomly perturbed, system by means of two diffusing chemicals: an activator that stimulates synthesis of an inhibitor, that, in turn, inhibits synthesis of the activator. Given the right reaction parameters and assuming that the inhibitor diffuses more rapidly than the activator, it is possible to establish a stable spatial pattern of activator and inhibitor concentrations. Presumably these chemicals can then induce spatial patterns of novel gene expression that lead to morphogenesis. Turing style mechanisms have been shown to operate in the formation of color patterns in reef fish (Kondo, 2002; Kondo & Asai, 1995; Kondo & Miura, 2010), and have been hypothesized to act in many other systems (Meinhardt, 1982; Murray, 2003; Sheth et al., 2012). A significant problem with Turing mechanisms is that the patterns they produce are very sensitive to parameter values and to field size. Murray (1982) showed that the parameter space for producing stable patterns for several variants of a Turing mechanisms are exceptionally small, and parameters often have to be specified to several significant digits in order to obtain the desired pattern. Changes in the size of the field on which a Turing system operates can also lead to different patterns (Madzvamuse, Gaffney, & Maini, 2010; Meinhardt, 1982; Murray, 1982, 2003). These findings stand in contrast to the fact that in development patterns are quite robust to variation in size as well as to a large amount of variation in the concentrations of morphogenetic gene products. Most animals and plants have a substantial amount of non-genetic (plastic) variation in size, yet pattern formation proceeds almost independent of size (Gonzalez, Kristensen, Morck, Boyd, & Hallgrímsson, 2013; Hanken & Wake, 1993; Lammers & German, 2002; Niklas, 1994; Polilov & Beutel, 2009; Quesada et al., 2011). Thus development exhibits a degree of robustness not seen in Turing-type mechanisms.

Studies on pattern formation in *Drosophila* embryos and imaginal disks suggest that much simpler mechanisms are at work there. Patterned gene expression in these systems appears to be controlled by a diffusion-gradient-threshold mechanism.

Transcriptional regulators act as diffusible morphogens that are produced at restricted locations. Their concentration declines with distance from the source. At a given threshold concentration they stimulate the transcription of a new transcriptional regulator that also diffuses away from that site of origin and stimulates another round of transcription at a given threshold value (Carroll, Grenier, Weatherbee, & From, 2004; Gilbert, 1997). A cascade, or rather, a network of transcriptional regulators sets up ever more localized and refined gradients of transcriptional regulators (Figure 6). These transcription factors, in turn, induce localized protein synthesis that leads to localized growth and differentiation of the cells of the developing embryo.

Gradients of morphogens are found throughout development and are involved in establishing axes of differentiation in developing limbs (Amthor, Christ, Weil, & Patel, 1998; Barna, Pandolfi, & Niswander, 2005; Cooper et al., 2011; Delgado & Torres, 2016; Lecuit & Cohen, 1997; Tickle, 2003), craniofacial features (Douarin & Creuzet, 2009; Evans & Francis-West, 2005; Trainor, 2005), digit differentiation (Dahn & Fallon, 2000; Suzuki, 2013), insect wing venation patterning (Bier, 2000; Blair, 2007), color pattern development (Carroll et al., 1994; Kondo & Asai, 1995; Monteiro, French, Smit, Brakefield, & Metz, 2001; Nijhout, 1980; True, Edwards, Yamamoto, & Carroll, 1999; Weatherbee et al., 1999; Zhang & Reed, 2016), and many others processes by which cells and tissues become different from one another. Diffusion gradients not only supply positional information (Wolpert, 1981; Wolpert, 1994), but can also be used to scale patterns to fields that vary in size and thus may provide a robustness mechanism for development. For instance, if a diffusion gradient is established by a mechanism that has a source at one end of a developmental field and a sink at the other end, then at steady-state there will be a linear gradient whose slope depends on the distance between source and sink, and specific concentration thresholds always are at a constant proportional fraction of the length of the field. This is an unlikely stabilizing mechanism, however. Barkai and Leibler (1997) have argued that diffusion gradients are probably never at steady-state because development progresses too rapidly. This implies that the local concentration of a diffusing morphogen is continually changing, even as the developmental processes induced by the morphogen gradient are proceeding. Robustness in specifying those processes involves a sometimes complex and dynamic set of feedback interactions; it is not simply a matter of "reading" the local concentration of a morphogen at some point in time (Jaeger, Irons, & Monk, 2008). Binding of a morphogen to its receptor can cause inhibition and upregulation of the receptor, which then sequesters the morphogen. This sharpens and steepens the gradient of the morphogen locally, and mathematical modeling indicates that it also makes the response less sensitive to individual differences and temporal fluctuations in the gradient (Barkai & Leibler, 1997; Cadigan, Fish, Rulifson, & Nusse, 1998; Eldar, Shilo, & Barkai, 2004). This feedback mechanism can therefore produce a robust, sharp, and predictable response to a shallow and variable gradient of morphogen (Jaeger et al., 2008). In Wingless signaling, the morphogen not only activates a response in receptive cells but also causes them to produce a short-range inhibitory signal that represses the same response in surrounding cells (Piddini & Vincent, 2009). Similar lateral inhibition mechanisms have been shown to operate in neurogenesis. Here an initially homogeneous group of neural progenitor cells is activated and initiate a neighbor-to neighbor signaling involving Notch and Delta, two transmembrane proteins in which Notch acts as a receptor and Delta as a ligand. When Delta-Notch binding is activated it induces

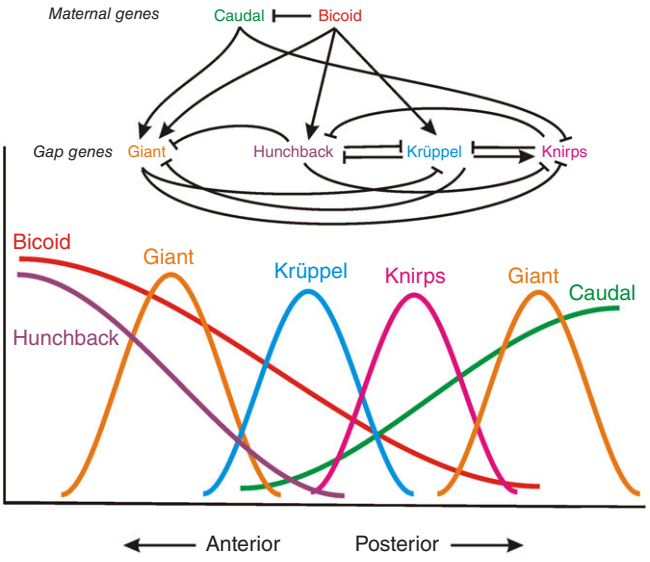

FIGURE 6 Patterning network in an early *Drosophila* embryo. The mRNA for the *bicoid* and *caudal* transcription factors are laid down as the egg is made and, after fertilization, stimulate expression of the so-called gap genes, which also mutually stimulate and inhibit each other's expression. *Caudal* is initially homogeneously distributed but is inhibited by *bicoid. Hunchback* is unusual in that at low concentrations it stimulates *krüppel*, but at high concentrations inhibits its expression. The gap genes form a banding pattern on the embryo that in turn controls a progressively more refined expression of subsequent genes that control formation of the segments

repression of Delta in surrounding cells, so that only one cell in a particular neighborhood remains with active Delta; that cell differentiates into a neuroblast cell that will generate neurons, whereas the surrounding cells will form ectodermal epithelial tissues (Meir et al., 2002). This mechanism works for communication among adjoining cells, but can extend over may cells via long filopodia that can interact with distant cells (de Joussineau et al., 2003). Lateral inhibition provides a robust mechanism by which single cells in an initially homogeneous cluster, can become differentiated and produce a novel tissue. A simple, (nondynamic) mechanism of robustness in morphogenetic signaling involves duplication and redundancy in the transcriptional regulators, so that if one is mutated, or its expression is defective, others maintain normal function (Osterwalder et al., 2018).

The morphogenetic gradients, peaks and valleys that are set up during development control downstream pathways that stimulate local growth, cell death, cell specialization and tissue differentiation. What is still missing in most if not all cases, is an understanding of how the "endpoint" is determined. How does a tissue, organ, or appendage "know" it has arrived at its final size and shape? It is this endpoint that gives development a goal-oriented appearance, both in normal development and regeneration. Is it that the development, growth and differentiation pathway is autonomous for each tissue and organ, so that each part of an organism is basically on "autopilot," as self-organization theory implies. Or is there some kind of centralized overall control that coordinates growth and development in different parts of the body so that the sizes and proportions always come out just right? Is there some kind of homeostatic set point that brings the overall size and shape to the correct endpoint? And how is that set point encoded? Some evidence for a set point-like mechanism comes from studies on imaginal disk regeneration in *Drosophila.* When a wing imaginal disk is damaged, it sends out a signal, probably an insulin-like growth factor (Colombani, Andersen, & Léopold, 2012; Garelli, Gontijo, Miguela, Caparros, & Dominguez, 2012), that both stimulates regeneration and inhibits metamorphosis by suppressing production of the molting hormone, ecdysone, until the wing disk is fully regenerated and cell division stops (Bryant & Levinson, 1985; Colombani et al., 2012; Stieper, Kupershtok, Driscoll, & Shingleton, 2008). A different kind of systemic regulation of wing size in *Drosophila* is shown by experiments that alter cell division patterns and induce clones of small or large cells in the developing wing (Neufeld, de la Cruz, Johnston, & Edgar, 1998; Weinkove, Neufeld, Twardzik, Waterfield, & Leevers, 1999). In such cases, the overall size and shape of the wing is unaffected, so it seems that wing size is independent of cell size or cell number (McCabe, French, & Partridge, 1997; Weinkove et al., 1999). Multiple mechanisms have been hypothesized for size regulation in the wing (Aegerter-Wilmsen, Aegerter, Hafen, & Basler, 2007; Affolter & Basler, 2007; Neto-Silva, Wells, & Johnston, 2009; Shingleton, 2010). Other examples of systemic control mechanisms over growth and size are evident in the control of overall body size and body-wing scaling in insects outlined in the next section.

5.5.1 | The regulation of size

Body size and shape (the relative sizes and dimensions of body parts) are the most distinguishing features of a species. A given species grows to the same size and shape in spite of many genetic and environmental insults. Lack of nutrients, unless fatal, simply slows growth until the final, species-specific, size is attained. Under-nutrition can stunt final size, of course, but not to a great extent: undernourished elephants will never grow to the size of a mouse, just smallish (but still very large) elephants. Size and shape are among the most robust features of animals. Mice are never mistaken for rats (or vice versa), no matter what mutations or environment they are exposed to, and shape abnormalities are sufficiently rare and remarkable, that they often end up in newspapers, museums, and research laboratories. With the exception of a few cases in insects (see below), nothing is known about the mechanism by which animals with determinate growth regulate when to stop growing. Much is known about how growth is controlled by growth hormone and insulin-like growth factors, but nothing about how "size" is regulated. That is, how the control of growth factors is integrated with a size-sensing mechanism so that growth stops when the "right" size is reached.

In multicellular animals, cells grow and divide only when stimulated by paracrine or endocrine growth factors or growth hormones. This implies that growth is not an autonomous property of a cell or tissue but is controlled systemically. The regulation of growth therefore depends on the regulation of growth promoting factors, and the regulation of size is a question of when to stop growing, or rather, when the growth factors are turned off, or when the receptor pathway for those growth factors is inactivated.

The mechanisms that control size are largely unknown but are beginning to be understood in insects, and involve various physiological and biochemical feedback systems. The simplest is found in the Hemiptera (True Bugs). These animals have stretch receptors in their abdominal muscles, and when these are stretched to a critical degree they send a message to the brain that starts secretion of the prothoracic tropic hormone (PTTH), which stimulates secretion of the molting hormone, ecdysone (Nijhout, 1979; Nijhout, 1984). Ecdysone causes the animal to stop feeding and induces the metamorphic molt that turns the larva into an adult. Adult insects (just like birds and mammals) do not grow, so the size at which the larva initiates the metamorphic molt determines the size of the adult. Larvae can be induced to metamorphose by an injection of saline, or even an air bubble, that expands the abdomen sufficiently to induce the hormone cascade, and this results in miniature adults (Nijhout, 1979).

This stretch-receptor mediated feedback mechanism appears to operate only in the Hemiptera. Lepidoptera and Diptera (and perhaps other insects) use a different mechanism to control body size. These insects rely on the fact that the tracheal system, a network of finely branching tubules that take atmospheric oxygen directly every cell in the body, is lined with cuticle, like the exoskeleton, and does not grow. As the larva grows in mass, it requires more oxygen, but the oxygen delivery system is fixed. Eventually the need for oxygen exceeds the delivery capacity of the tracheal system. At that point, referred to as the critical weight, the secretion of juvenile hormone (JH) stops and its level in the blood begins to decline (Callier & Nijhout, 2011; Nijhout, 1975; Nijhout & Williams, 1974). During the last larval stage JH inhibits secretion of PTTH and ecdysone (Nijhout & Williams, 1974; Rountree & Bollenbacher, 1984). Once JH disappears and this inhibition is relieved, PTTH and ecdysone secretion begin, which stop growth and initiate the metamorphic process. Rearing larvae under hypoxic conditions initiates a premature metamorphic molt and results in miniature adults (Callier & Nijhout, 2011, 2013). Thus, in both cases a feedback loop from a factor that remains constant (stretch receptor length or tracheal system volume), within a growing body, triggers a hormonal cascade that stops growth. Spectacular and future challenges for homeostasis are the regulation of other kinds of higher level properties, like the period of the circadian clock, how system function is maintained in the presence of continual massive turnover of its component parts, and how perfect bilateral symmetry is generated and maintained.

5.6 | Complex homeostasis in metabolic systems

Mathematical modeling of several large metabolic systems has revealed multiple homeostatic mechanisms that serve to stabilize function against both temporal variation in input, and individual variation in mutational load of genes for enzymes in the system. One carbon metabolism (OCM), illustrated in Figure 3, is a key metabolic network that contains the initial steps for de-novo purine and pyrimidine synthesis, as well as a host of methylation reactions such a DNA methyl transferase (DNMT). Defects in this metabolic network are associated with a broad range of diseases, ranging from megaloblastic anemia to spina bifida, various cancers, and cardiovascular disease. Such defects can arise due to mutations in genes that code for the enzymes in the network, or due to nutrient and vitamin deficiencies. Nutrients like amino acids (methionine, glycine, serine) are used as sources of methyl groups, and the B vitamins (folic acid, B_6, and B_{12}) are cofactors for several of the enzymes in OCM (Gregory III et al., 2013; Nijhout et al., 2009; Reynolds, 2006; Stover, 2004). The thymidylate synthase (TS) reaction is the rate limiting step for DNA synthesis (and cell division), and, together with dihydrofolate reductase (DHFR), the next enzyme in the pathway, is a target for anticancer drugs. Because of these associations, both the structure of this network, as well as the kinetics of the enzymes, have been exceptionally well studied. This makes it possible to develop and validate mathematical models of the system, which allows us to study the effects of mutations, and of variation in nutrients and vitamins (Nijhout et al., 2015).

The OCM network is a complex set of interlocking cycles, and is criss-crossed with allosteric interactions by which metabolites either activate or inhibit enzymes at various locations in the network (indicated by red arrows in Figure 3). These regulatory interactions serve to stabilize various critical components of the network and make the operation of the network robust and relatively insensitive to precise values of the kinetic parameters.

With a mathematical model one can simulate hourly and daily fluctuations of amino acid input with meals (Nijhout et al., 2008). Figure 7 shows the responses of several metabolites and fluxes to a simulated daily meal regimen. With variation in amino acid input, some metabolite concentrations fluctuate wildly, as do many of the reaction fluxes. Some fluxes even change direction (Nijhout et al., 2008) (Figure 7a). There are four variables, however, that remain remarkably constant: the TS and AICART reactions, required for de-novo synthesis of pyrimidines and purines, respectively, the DNA methylation reaction flux, and the concentration of glutathione (Figure 7c). This system therefore has all the characteristics of a homeostatic system: one or a few variables are kept stable by large variation in others, which compensate for variable environmental input (amino acid input in this case). The four variables that are kept stable make sense. They are DNA synthesis, which should not be impaired by variation in nutrition, and DNA methylation, which needs to keep up with DNA synthesis. Glutathione is the main endogenous antioxidant. A significant decrease in concentration would impair the ability to deal with oxidative stress that accompanies metabolism, as well as the detoxification of both endogenous and exogenous toxins. These four variables can be thought of as the critical phenotypes of the OCM network, since special feedback interactions evolved to stabilize them. The fact that the allosteric feedback reactions are responsible for this stability is easily verified by eliminating one or more from the model (Nijhout, Reed, Anderson, et al., 2006). An example is shown in Figure 7d where the product feedback inhibition of DNMT is eliminated with the result that the rate of the DNMT reaction now fluctuates strongly with amino acid input.

The same allosteric feedback reactions that stabilize certain fluxes in the OCM network to variation in input also stabilize them against mutations in critical enzymes in the pathway. In the mathematical model of a biochemical reaction system, a mutation that alters the activity or the expression level of an enzyme can be modeled by altering the enzyme's V_{max}. Figure 8 illustrates several examples of how a critical reaction flux (the TS or the AICART reaction) varies with variation in the activities of various enzymes in the OCM network. The x and y axes represent enzyme activities and the z axis is the "phenotype."

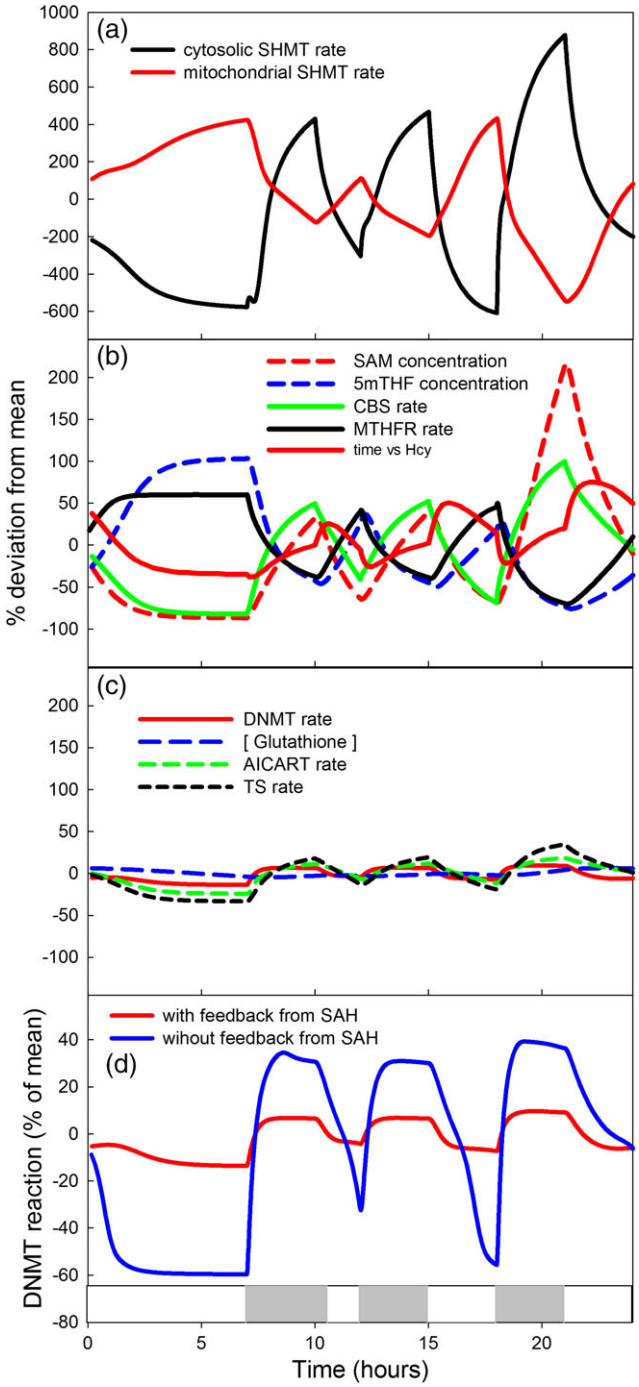

FIGURE 7 Sensitivity and homeostasis in OCM. Gray bars below (d) show the temporal pattern of amino acid input into blood compartment of the model shown in Figure 3, to match known fluctuations of amino acids with meals. This amino acid influx produces fluctuations in both fluxes and metabolite concentrations. (a) The reaction of SHMT interconverts serine and glycine varies greatly, and can even change direction. (b) Metabolite concentrations and fluxes can change more than twofold in the course of a few hours. (c) The rates of the TS, AICART and DNMT reactions, and the concentration of glutathione, are quite stable and are little affected by variation in the other enzymes and metabolites in the system. Indeed, these four factors are stabile *because* of fluctuations in other parts of the system that counteract the effects of variation in amino acid influx, and act as homeostatic regulators. (d) Removal of one of these regulators (product inhibition by SAH), destabilizes the DNMT reaction, which now becomes much more sensitive to amino acid fluctuations. Based on (Reprinted with permission from Nijhout et al., (2008))

The graphs in Figure 8 thus show how the value of the phenotype depends on the activities of the enzymes. This "phenotypic surface" is clearly nonlinear. For much of its extent it is relatively flat and orthogonal to the phenotype axis. This means that variation in the enzymes on the *x* and *y* axes have very little effect on the value of the phenotype. In other words, the phenotype is robust to this variation. Now one might argue that the enzymes shown on the *x* and *y* axes may not be expected to have much of an effect on the property shown on the *z*-axis. To test that it is the allosteric interactions that provide this homeostatic property one can remove one or more of them in the model. The right hand column in Figure 8 shows that removal of one of

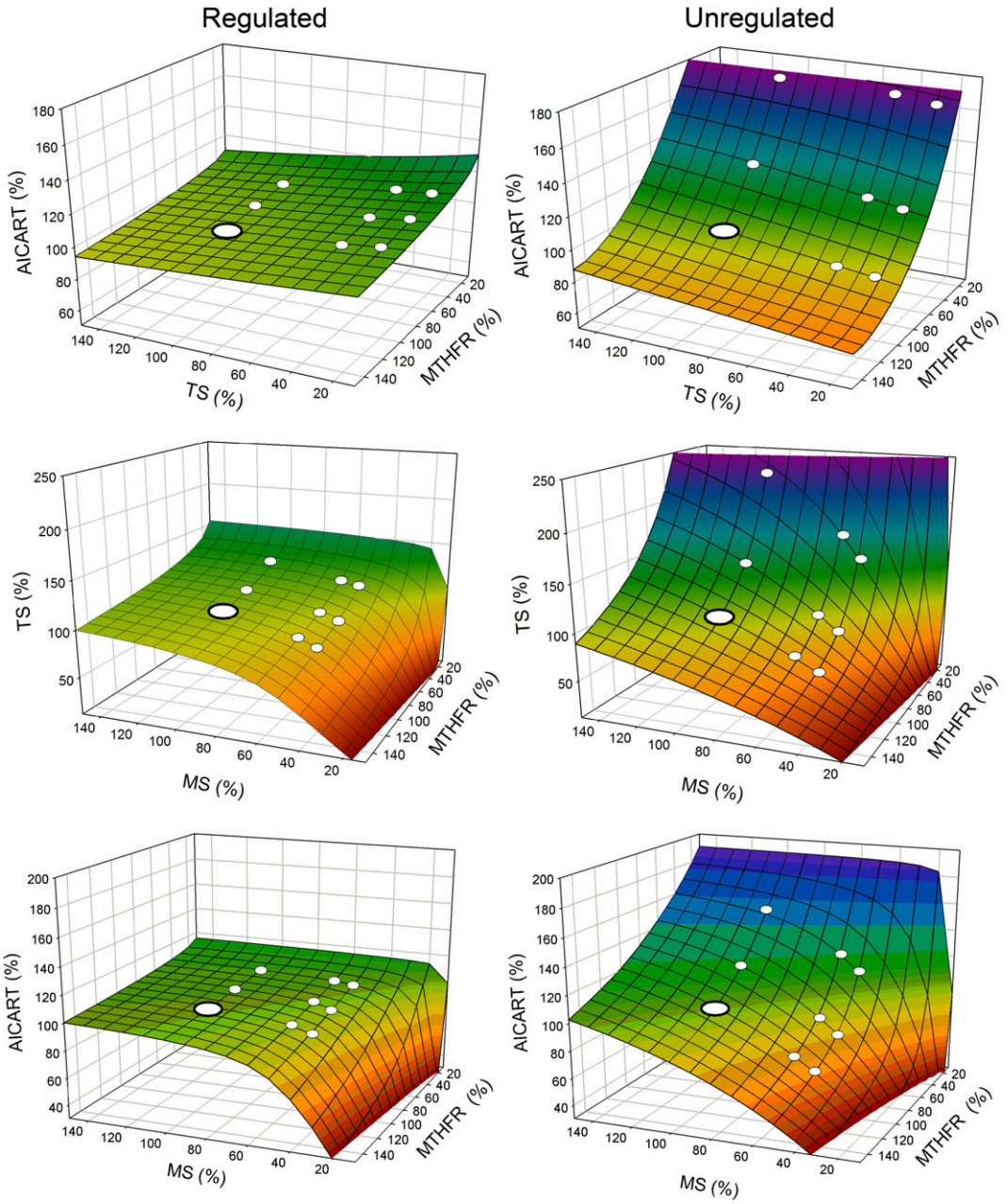

FIGURE 8 Stability of the TS and AICART reactions to polymorphisms in MS, MTHFR and TS genes. The rates of the reactions as pairwise functions of variation in the activities of MTHFR, MS and TS, scaled relative to the wild-type values. The wild types are indicated by the large circles. The small circles show the locations of genetic polymorphisms, taken from Table 1. The left column shows the phenotypic landscapes with all allosteric regulatory interactions in place. The right column shows the same phenotypic surfaces after removing the allosteric regulation of MTHFR by SAM (see Figure 3). In each case, removing the allosteric regulation causes the phenotypic surface to be "tilted," so that polymorphisms that produced nearly identical phenotypes (z axis), and were "cryptic" with respect to phenotypic variation, now produce different, abnormal, phenotypic values

the allosteric interactions severely changes the shape of the phenotypic landscape so that it is no longer perpendicular to the z-axis, so variation in enzyme activity now has a big effect on the phenotype.

It is unlikely that the dense network of allosteric regulatory interactions shown in Figure 3 arose all at once. Presumably the interactions evolved gradually, one by one, to improve stability of the critical reactions against different environmental or genetic variations. In some cases it is possible to deduce the order in which some reactions are likely to have evolved. The DNA methyl transferase (DNMT) reaction is under allosteric control by its substrate SAM. SAM activates cystathionine β-synthase (CBS) and inhibits methylene tetrahydrofolate reductase (MTHFR) (see Figure 3). In the presence of both regulations, the DNMT reaction is very insensitive to the input of methionine, the primary methyl donor for the reaction (Nijhout, Reed, Anderson, et al., 2006). Decreasing methionine input from 140 to 10 μM/hr only reduces the rate of the DNMT reaction from 150 to 130 μM/hr. In the absence of both regulations the DNMT reaction declines strongly with declining methionine input, dropping from 150 to 40 μM/hr (Figure 9). Adding one or the other of the two allosteric effects significantly improves

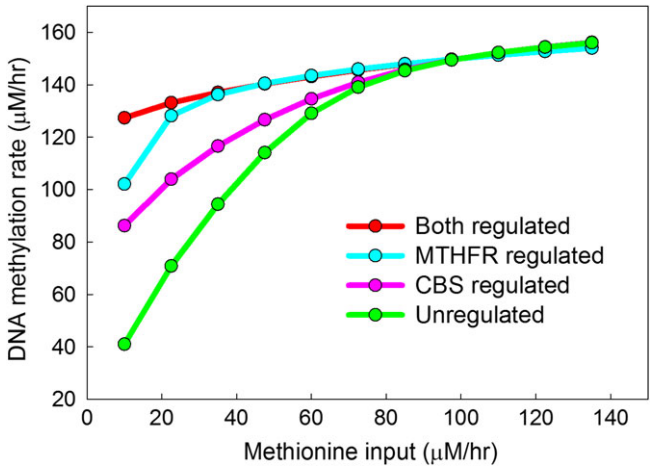

FIGURE 9 Multiple stabilization of the DNMT reaction. In addition to product inhibition (Figure 7(d)), the DNMT reaction is also stabilized by allosteric regulation of CBS and MTHFR by SAM (shown in Figure 3). In the absence of these two regulators the DNMT reaction rate is nonlinearly proportional to methionine input, which supplies methyl groups. When both CBS and MTHFR are regulated the DNMT reaction rate is quite insensitive to methionine input. The reason is that these two regulations enable more methyl group input from the folate cycle. Regulation of MTHFR alone provides much more stability than regulation of CBS alone. This implies that the regulation of CBS evolved first, because had regulation of MTHFR been established first there would be little fitness benefit of adding regulation of CBS. The improvement of DNMT is strongest at low methionine input, suggesting that the regulations may be an adaptation to prolonged periods of protein deficiency. Based on (Reprinted with permission from Nijhout, Reed, Anderson, et al. (2006))

stability of the DNMT reaction to variation in methionine input. Adding the MTHFR regulation has a much stronger effect than adding the regulation of CBS. This suggests that the regulation of CBS probably evolved first, because the marginal effect of adding it to a pre-existing regulation of MTHFR is very small. Overall the regulations have their strongest effect at low methionine inputs, which suggest that they might have evolved to stabilize DNMT against periods of low protein nutrition that arise seasonally.

5.6.1 | Genetic polymorphisms and cryptic genetic variation

There are significant polymorphisms in many of the genes for enzymes in OCM. Genetic epidemiology studies show that these polymorphisms are associated with various diseases, although the associations are often weak and depend on the population being studied. Many of the polymorphisms occur at high frequencies in some populations and, in cases where the actual functional effect of the polymorphism has been studied, they can have a large effect on the activities of the corresponding enzymes. Table 1 shows examples of some of these polymorphisms with their functional effects and frequencies in selected populations. The high frequencies of the defective alleles and their large effect on the activity of the encoded enzymes is remarkable, and it might be surprising that they are only weakly associated with disease. When these polymorphisms are plotted on the relevant phenotypic landscapes it is clear that they lie on the homeostatic plateaus (Figure 8). Thus, although these mutations can have a very large effect at the functional level, on enzyme activity, they have little effect at the phenotypic level.

TABLE 1 Common large-effect mutations in one carbon metabolism, their effects on the activities of the respective enzymes, and frequencies in selected populations[a]

	Mutation		Frequency	
Enzyme	vitamin cofactor		activity wrt wild type	
MS	B_{12}	A2756G	50%	9% (C) 16% (US) 20% (EU)
MS		D919G	60%	17% (J) 55% (US)
MTHFR	B_2 B_9	C677T	30%	51% (I) 34.5% (ME) 35% (US)
MTHFR		A1298C	68%	33% (I) 33% (US)
TS	B_9	2rpt/3rpt	42%	48% (US) 40% (EU) 8% (C)
TS		1494del6	24%	76% (US) 33% (C)
CBS	B_6	M173V	38%	–
CBS		A226T	13%	4.5% (AA)
CBS		R548Q	60%	0.6% (S)
CBS		T191M	10%	14–75% (H)

[a] For references see Kennedy et al. (2012) and Nazki, Sameer, and Ganaie (2014).
Enzyme—MS: methionine synthase; MTHFR: methylene tetrahydrofolate reductase; CBS: cystathionine β-synthase; Frequency—AA: African Americans; C: China; EU: Europe; H: Hispanics; I: Italy; J: Japan; ME: Middle East; S: Spain; US: United States.

This phenotypic stability depends on the operation of the allosteric regulatory mechanisms, as shown above in Figure 8. This implies that mutations can accumulate within the homeostatic region because they have little effect on the phenotype and thus are not, or only weakly, selected against by natural selection. Mutations that have little or no effect on the phenotype are referred to as "cryptic genetic variation." If a mutation in the regulatory mechanism alters the slope of the homeostatic plateau (right-hand column in Figure 8) it would increase the functional effect of a polymorphism and now the mutation could be deleterious. This is what is meant by a mutation giving a "predisposition" to disease: the phenotype is normally on a homeostatic plateau, but maybe near an edge, so that if anything disrupts the homeostasis, the mutation's deleterious effects become evident.

5.7 | Equivalence of genetic and environmental factors

It is common to think of environmental robustness and genetic robustness as separate things (Masel & Siegal, 2009; de Visser et al., 2003; Gibson, 2002; Gibson & Wagner, 2000; Lehner, 2010; Stewart et al., 2012; Wagner, 2000, 2005; Wagner, 2008; Wagner, Booth, & Bagheri-Chaichian, 1997), but that is not necessarily the case. This dichotomous view emerged primarily from studies that were particularly interested in one or the other, and from the fact that environmental and genetic variation are incommensurate and difficult or impractical to manipulate simultaneously. This is not to say that the mechanisms that make phenotypes robust to environmental variation are necessarily different from those that make them robust to genetic variation, and we will see examples below in which the same mechanism stabilizes phenotypes against both genetic and environmental variables.

Genes and environments are typically thought to affect a phenotype through different pathways and their combined effects are analyzed statistically as a gene–environment interaction (Falconer & Mackay, 1996; Lynch & Walsh, 1998). Mathematical models of a system make it possible to integrate genetic and environmental factors (if their effect on the kinetics of a system or its components are understood). They make it possible to study the causal chain by which gene–environment interactions emerge, and to study whether different aspects of the operation of a system are more or less subject to environmental variation than genetic variation. Mathematical models generally require data generated by experimentalists, as well as data from proteomic, metabolomic and transcriptomic studies to parametrize a model. Models, therefore, provide a natural platform for integration among the various 'omics. A useful mathematical model is one that helps us to better understand how a system works, or resolves a puzzle or disagreement about mechanism, or predicts the outcome of an experiment. Ideally models are developed in collaboration with, and benefit from reciprocal illumination among, experimentalists and modelers.

The effects of specific environmental factors can be explicitly taken into account in a mathematical model of a system. For instance, vitamin B_{12} is a cofactor for the enzyme methionine synthase (MS). A B_{12} deficiency reduces the activity of the enzyme in the same way a mutation would, so the axis of MS activity shown in Figure 8, could be interpreted to represent the effects of mutations as well as the effects of variation in vitamin B_{12} level. Variation in nutrient input can be explicitly modeled (e.g., Figure 7), and its interaction with genetic variation can be plotted as a phenotypic landscape (e.g., Figure 10a). Environmental factors can also disrupt a homeostatic system. Staying with the example of a vitamin B_{12} deficiency in OCM, Figure 10b illustrates how the phenotypic landscape for the DNMT reaction changes with such a vitamin deficiency. The landscape "tilts" and is no longer homeostatic for variation in TS. This implies that a vitamin deficiency can unmask previously hidden cryptic genetic variation and can cause defective genes whose effects were masked by the homeostatic mechanism to contribute now to phenotypic variation.

Similar homeostatic mechanisms are known to operate in other cellular and biochemical systems. In dopamine and serotonin metabolism, for instance there are four feedback interactions that stabilize dopamine and serotonin in the synapse or near a varicosity. The first is provided by reuptake transporters that remove the neurotransmitter from the synapse into the presynaptic terminal, where it is repackaged and readied for rerelease (Best, Nijhout, & Reed, 2009; Best, Nijhout, & Reed, 2010a; Best, Nijhout, & Reed, 2010b). The second is an autoreceptor on the presynaptic neuron that inhibits synthesis and release of the neurotransmitter if its concentration in the synapse rises (Best et al., 2009, 2010a, 2010b). The third is the fact that tyrosine hydroxylase and tryptophan hydroxylase (TPH) show substrate inhibition (Best et al., 2009, 2010a, 2010b). The fourth is product inhibition of the TPH reaction by cytosolic serotonin. All four mechanisms stabilize the serotonin concentration in the synapse. The phenotypic landscape for extracellular serotonin as a function of TPH, the first enzyme in the biosynthetic pathway, and the serotonin reuptake transporter (SERT) is shown in Figure 11. There are large-effect, common polymorphisms in both genes, and, as in the cases of OCM illustrated in Figure 8, these fall on the homeostatic plateau for extracellular serotonin (Figure 11). Low serotonin is associated with depression (Feldman, Meyer, & Quenzer, 1997). Some of the polymorphisms put phenotypes at the edge of the homeostatic plateau, and it would not be unreasonable to suppose that this implies a stronger predisposition to depression than would be found in the wild type.

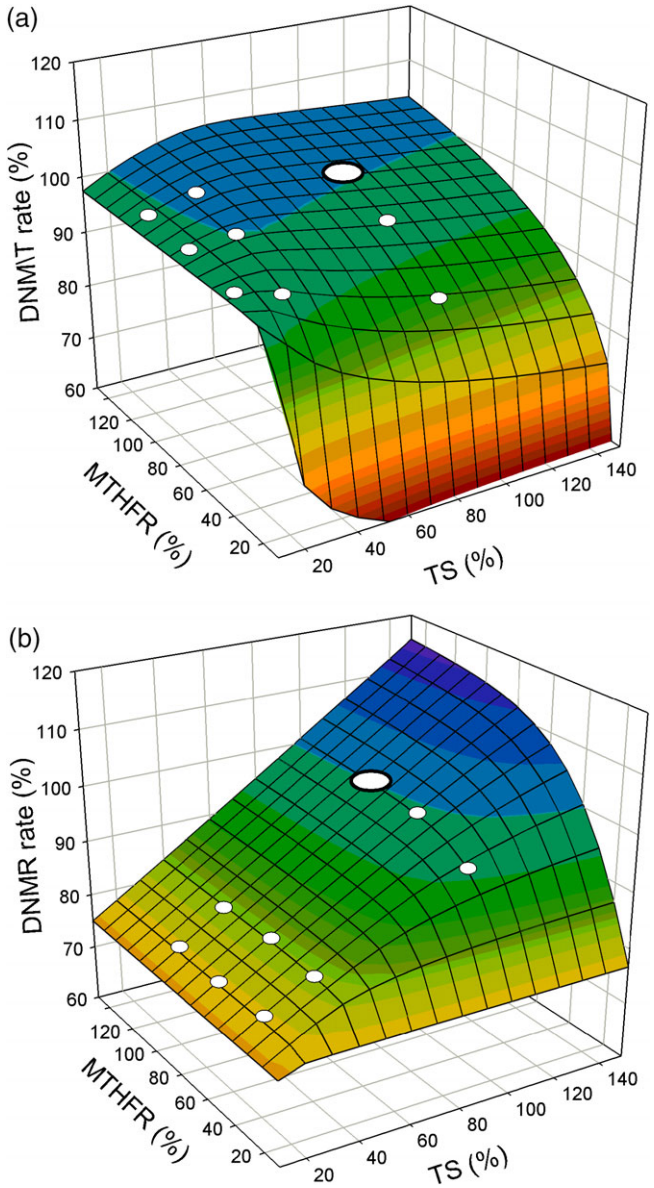

FIGURE 10 Environmental destabilization of homeostasis. Stability of the DNMT reaction rate to polymorphisms in the MS and MTHFR genes, scaled relative to the wild-type values. The wild types are indicated by the large circles. The small circles show the locations of genetic polymorphisms, taken from Table 1. (a) Stability under normal concentration of vitamin B_{12}. (b) A vitamin B_{12} deficiency causes a "tilt" in the phenotypic landscape, and mutations that were cryptic with respect to phenotypic variation, now produce different, abnormal, DNMT phenotypes

5.8 | Biological variation, precision medicine, and systems population models

We discussed above the exceptional amount of variation in biological systems; no two individuals are alike in genetic makeup, gene expression levels, or environmental exposures. Yet this variation is usually ignored and measures of biological variables are given as means with standard deviations, where the mean represents the value for some idealized or "average" individual. Traditionally, medicine treats this average individual. It is the goal of precision medicine to treat each person by taking in account his or her individual characteristics. For several reasons, this is a daunting task. Of course, an individual's genome is easy to obtain, but gene expression levels vary by 25% from individual to individual (Oleksiak et al., 2002; Sigal et al., 2006), and the methylation patterns on the genes depends on life history. Furthermore, it is difficult to even imagine accurate and useful ways of quantifying life history of environmental input, including diet and exercise.

Biological variation poses analogous issues for those of us who make mathematical models of biological systems in order to understand how the systems work. For simplicity, we'll consider a system of ordinary differential equations (ODEs) that models a biochemical network, although the ideas and methods can be used for gene regulatory networks, gene-metabolic systems, physiological and developmental systems. In the model, one must choose K_m and V_{max} values for each enzyme, and constants that represent the strengths of allosteric interactions or rates of transport between compartments. Such "constants" have a wide range of values in the literature (often over two orders of magnitude) reflecting the varied circumstances in which

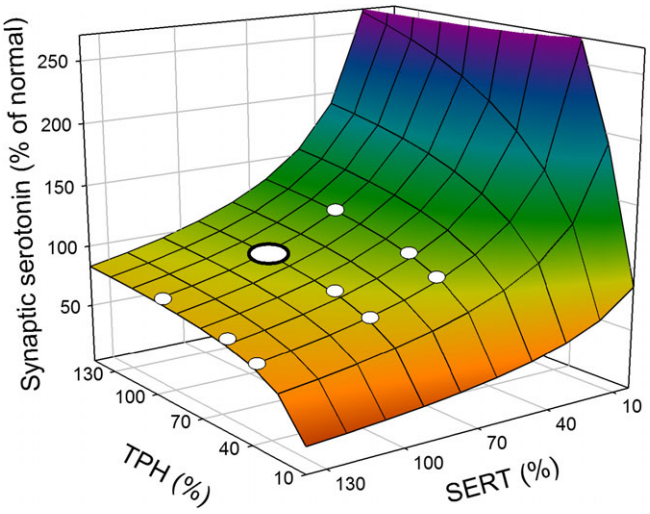

FIGURE 11 Stability of extracellular serotonin to polymorphisms in serotonin metabolism genes. The extracellular serotonin concentration as a function of variation in tryptophan hydroxylase (TPH) and the serotonin reuptake transporter (SERT), scaled relative to the wild-type values. The wild type is indicated by the large circle. The effects on enzyme activity of polymorphisms in genes for TPH and SERT are indicated by small circles. Polymorphisms shown for TPH are C2745A and R441H, which reduce activity to 65% and 40% of normal, respectively. Those for SERT are the short repeat allele (*s*) in the regulatory region of the gene and the I425V snp, which reduce activity to 33% of normal and increase activity to about 150% if normal, respectively

they were measured. So, for each constant one chooses an average or representative value. One then has a mathematical model for an "average" or idealized biochemical network and one can perform in silico experiments with the model, compare the results to extant data, and investigate mechanism. But this avoids dealing with the enormous biological variation between individuals.

We address both the biological issues and the mathematical issues by making **system population models** of biological systems. This type of model was introduced in (Duncan, Reed, & Nijhout, 2013a; Nijhout & Paulsen, 1997), and was reviewed in the mathematics literature by Swigon (2012). For a deterministic ODE model of a metabolic or physiological system, a system population model is constructed as follows. Each parameter has "normal value." A new value for that parameter is selected from a probability distribution whose mean is the normal value. This is done independently for all (or a subset of) the parameters. The model is then run to equilibrium and the parameter values, metabolite concentrations, and fluxes are recorded. That is one virtual individual. This procedure is repeated 10,000 times to produce a database of 10,000 virtual individuals, each with different parameters, concentrations, and fluxes. But in each individual, the concentrations and fluxes depend on the chosen parameters through the same mechanism (the ODEs). Below, we briefly discuss how population models have been used.

1. Traditional statistical analysis. Since one has a large database of virtual individuals, one can utilize traditional statistical analyses to find new correlations, analyze scatterplots, and find individual outliers. The difference is that since the data all comes from the same mechanistic model, one can conduct in silico experiments to understand the mechanisms that produce the correlations. We used this in Duncan et al. (2013a) and Duncan, Reed, and Nijhout (2013b) to show that plasma homocysteine is controlled by the non-liver tissues, not by the liver. Of course, one must compare one's virtual database to extant databases to verify that the model is good. We compare model distributions of plasma folate and homocysteine to distributions of actual human populations obtained from the NHANES database in Duncan et al. (2013a) and Nijhout et al. (2015). The model produces a population distribution of these variables with an excellent fit to human population data. Bayesian analysis of virtual population data, informed by a deterministic model, can also be used to identify candidate genes for particular traits or specific deviations from the wild type (Thomas et al., 2009).
2. Predisposition to disease. In Figure 11, all the polymorphisms of TPH and SERT lie on the homeostatic plateau where serotonin in the extracellular space does not vary much. These are for the "average" individual. Several polymorphisms are close to the edge of the cliff where serotonin drops rapidly. Individuals with those polymorphisms are predisposed to low serotonin, because if one varies all the parameters the shape of the homeostatic plateau will change, and for some individuals a polymorphism may no longer fall on the plateau. Such individuals will have very low serotonin. As before, an analysis of the virtual population may identify the particular combinations of parameter values that make individuals more or less susceptible to develop low serotonin. An example of such a study, for individual variation of dopamine levels, is shown in Figure 5 of Nijhout et al. (2017).

3. Relating deterministic models to biological data. Imagine an experiment where a experimenter changes the input to a biochemical network for a short period of time and then measures the values of a particular concentration over time. If the experiment is repeated several times, somewhat different curves will be obtained, and the experimenter will report the mean curve and the standard deviations. In a system population model, each different choice of parameters (individuals) will produce a different curve. It is this family of model output curves that should be compared to the family of experimental curves, not just the average model curve to the experimental mean curve, because there is real biological information in the dispersion of the curves. Thus, system population models are the right way to compare mechanistic ODE models to real biological data.
4. Identifying important subpopulations. A systems population model can be "treated" with a drug, or given a specific nutrient or vitamin deficiency, and the resulting population can be compared with a untreated population (Nijhout et al., 2017). Not all individuals will respond similarly, and statistical analyses of the two populations can be used to identify genetic make-ups (i.e., specific combinations of parameter values) that make individuals particularly sensitive, or resistant, to the treatment. The parameter makeup of those virtual individuals can then be used to determine which critical values need to be measured in real individuals to identify each subpopulation, and perhaps identify individuals who are likely to be good or bad responders.
5. The medical importance of the inverse problem**.** Consider the experiment described in (3) above, or consider the following example. We have three groups of mice, normal mice, obese mice, and depressed mice. The experiment consists of stimulating serotonin release and measuring the concentration of serotonin in the extracellular space over time, after the stimulation, as in Wood et al. (2014). Under repeated trials (repeated mice) for each group one obtains a family of curves. Those families of curves are (somewhat) different for the three groups. One wants to know what that tells us about how the parameters differ between the groups, because that is information about the internal mechanisms that are hard to measure directly. Given a large set of experimental curves, the inverse problem is to use those data to gain information about the distributions of parameters. The traditional methods described in Swigon (2012) cannot be used because those methods assume that variable distributions in the experiment are normal, and that is far from true both in experiments and in our system population models, where we find highly skewed as well as bimodal distributions that cannot be rescaled to normal. So this question requires new mathematical approaches. Virtual populations produced by system population models can provide a platform for testing such new mathematics, because in such populations everything is known: the topology of the network, the kinetics of the interactions, the values of all parameters, and the values of all variables, for each individual. Because of pervasive nonlinearities in real biological systems, none of the currently available mathematical techniques are able to reconstruct parameters and network topology from the data.

We close by giving an example that shows how difficult it is to draw firm conclusions from statistical analyses. Figure 12 shows scatterplots that relate several metabolites and flux rates in in OCM, partitioned into two subpopulations with high (red) and low (black) blood homocysteine. Homocysteine (Hcy) is a very reactive amino acid that is normally maintained at very low concentrations, and elevated levels of Hcy are recognized as a risk factor for cardiovascular disease (Carmel & Jacobsen, 2001). It is therefore important to understand in what way subpopulations with high and low plasma Hcy levels differ. One can see that each pair of variables is correlated. But the correlations are completely different in the high Hcy subpopulation and the low Hcy subpopulation! This is not an anomaly: the correlations between variables depends on the context in which they are measured. This what one would expect in a highly nonlinear, complicated system, that is crisscrossed by regulatory mechanisms, and serves as a reminder of how difficult it is to understand how biological systems work, and how misleading it can be if one uses a selected subset of a population to deduce parameters for a model. There's an interesting and difficult issue here and not just for mathematical modelers. Suppose a biologist measures two variables in a rat, and finds a correlation. How does she know that those rats are representative and do not come from a special subpopulation?

6 | CONCLUSION

All organisms walk the tightrope between stability and change. In this review, we have focused on the homeostatic mechanisms that produce homeostasis and robustness. On the genetic and metabolic levels, these mechanisms are typically a myriad of allosteric influences that protect some fluxes and metabolites against mutations and changes in environmental inputs. These mechanisms are diverse, interesting and sometimes complicated, but the properties that are homeostatic are relatively simple variables such as metabolite concentrations or fluxes. Physiological and developmental homeostatic and robustness mechanisms are more complicated because the properties that are homeostatic are themselves emergent properties of complex interacting systems. Because these systems have features that operate simultaneously at many levels of biological organization,

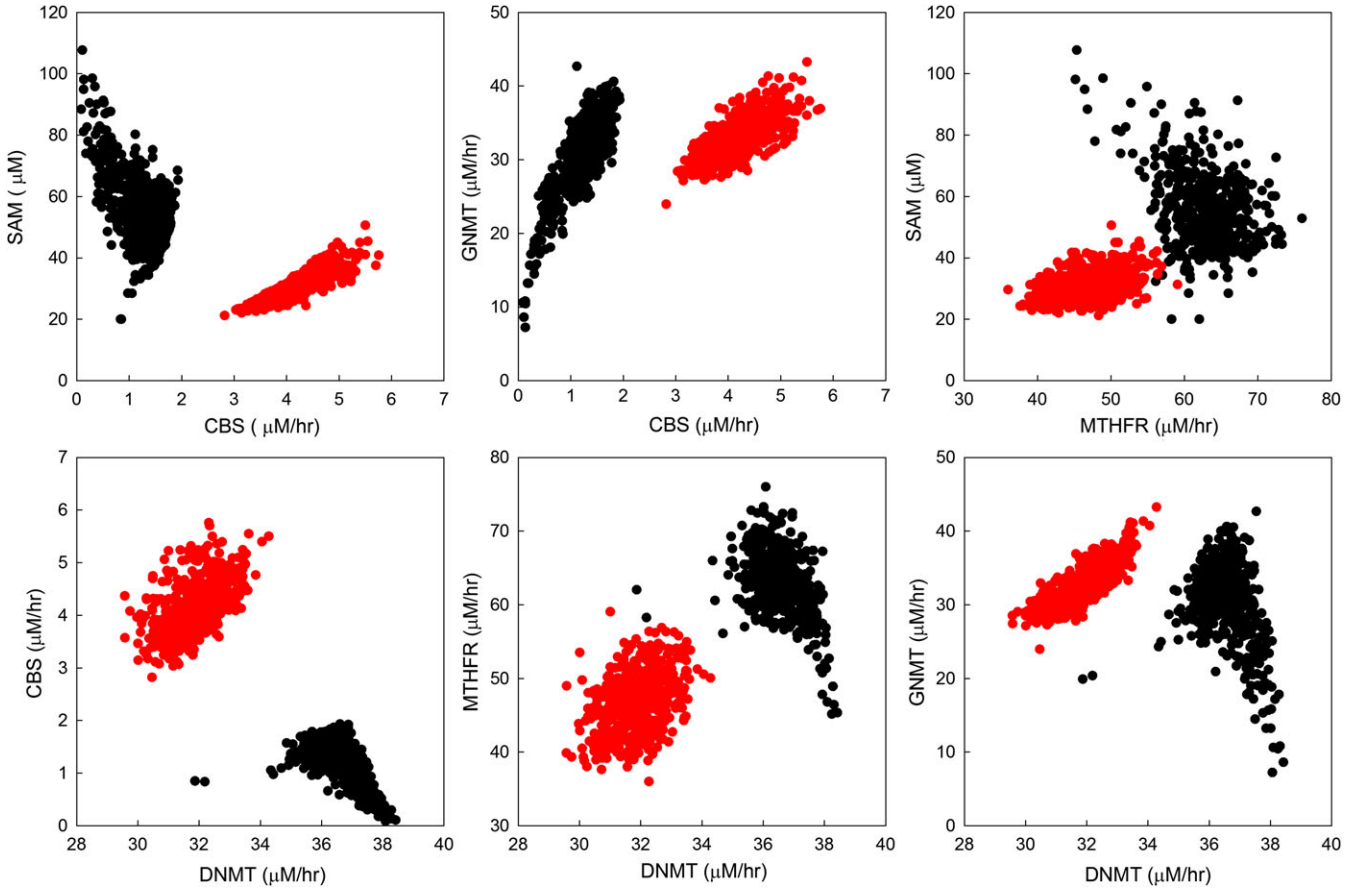

FIGURE 12 Scatterplots relating variation among several metabolites and fluxes in two subpopulations with high (red) and low (black) plasma homocysteine. Homocysteine (Hcy) is a highly reactive and toxic amino acid, produced in the methionine cycle, normally kept at very low concentrations. An elevated level of Hcy in the blood is a risk factor for cardiovascular disease. In each case the variables are clearly correlated, but the correlations are very different for the two subpopulations. The many nonlinearities and allosteric interactions in OCM cause the large differences in the associations among variables in subpopulations, which differ in slopes and elevations

from the molecular to the organismal, it is much more daunting to study and understand the causal mechanisms underlying homeostasis of higher level properties in developmental, neurological, and physiological systems.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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