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New views on the selection acting on genetic polymorphism in central metabolic genes

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Abstract

Studies of the polymorphism of central metabolic genes as a source of fitness variation in natural populations date back to the discovery of allozymes in the 1960s. The unique features of these genes and their enzymes and our knowledge base greatly facilitates the systems-level study of this group. The expectation that pathway flux control is central to understanding the molecular evolution of genes is discussed, as well as studies that attempt to place gene-specific molecular evolution and polymorphism into a context of pathway and network architecture. There is an increasingly complex picture of the metabolic genes assuming additional roles beyond their textbook anabolic and catabolic reactions. In particular, this review emphasizes the potential role of these genes as part of the energy-sensing machinery. It is underscored that the concentrations of key cellular metabolites are the reflections of cellular energy status and nutritional input. These metabolites are the top-down signaling messengers that set signaling through signaling pathways that are involved in energy economy. I propose that the polymorphisms in central metabolic genes shift metabolite concentrations and in that fashion act as genetic modifiers of the energy-state coupling to the transcriptional networks that affect physiological trade-offs with significant fitness consequences.

Keywords

life history; natural selection; fitness; energy sensing; metabolites; longevity

Introduction

The question of selection acting specifically on metabolic genes and their polymorphisms emerged soon after allozymes came to occupy center stage in evolutionary genetics in the late 1960s.¹ Full annotated genome sequences now present the opportunity to study any gene, yet the metabolic genes remain a special category that often appear enriched in studies of selection.² Now with next-generation sequencing (NGS) and the high-throughput of the many “-omics” technologies, it is relatively easy to amass considerable supporting information on important features of metabolic pathways—not only the gene sequences, but also the relative abundance of transcripts, proteins, and metabolites. We know that not only

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do these genes segregate for amino acid polymorphisms, but there is also an abundance of single nucleotide *cis*-acting expression variation in most metabolic genes. With the current ability to subject population genetic data to sophisticated population models, we have probably reached the limit in inferring historical selection in a model-dependent context. The current question about metabolic genes is whether the study of selection on their polymorphisms can be placed in a larger functional context rather than simply a list of unrelated single genes with unconnected and isolated functions.

This review speculates on the phenotypes that shape the genetic variation in the genes of central metabolism within natural populations. It draws upon several interconnected areas of relevance to the question of selection, such as aging research, signaling systems, and systems biology. There are many reviews that cover these areas, and as such there is an effort to direct the reader to a few reviews that allow entry into their content and tie into the discussion of selection on variation. My review possesses a bias towards *Drosophila* work, since this is a model with which I work. The emphasis is about the functional polymorphism at the structural and *cis*-expression levels that acquire appreciable population frequencies in these genes. Here I speculate about how this functional variation is translated into variation in phenotypes that have direct fitness consequences.

Common features of central metabolic genes

It is important to first emphasize the common features of metabolic genes and proteins that set them apart and lend them to the study of adaptive selection. First, this is an ancient group of highly conserved genes and pathways,³ where recognizing homologous sequences from whole genomes is unambiguous. They are so conserved that in yeast a high proportion of human genes can be substituted without notable reduction in growth.⁴ Second, these enzymes rank among the top 1–2% in expression and protein abundance,⁵ and this is also reflected in nucleotide variation, where the codon usage bias of the central metabolic genes ranks in the top 1% among all genes in *Drosophila*.⁶ Given that there are considerable fitness costs to protein expression,^{7–9} we might expect mutations that affect the fine-tuning of expression in the metabolic enzymes to possess fitness variation. Furthermore, while the causes of codon bias are many,^{10–12} the selection for optimal codon usage that is associated with translational efficiency is nonetheless clear and implies that translational efficiency is important in metabolic genes. The high expression of some metabolic proteins may have also played a part in their cooption into other roles unrelated to housekeeping functions. For no other group do we possess the wealth of knowledge on catalytic function, regulation, and interaction, as well as the unparalleled historical work on catalytic features of the many key pathway members.¹³ Beginning in the early 20th century, there has been a long history of their descriptions as implicated in diseases of inborn metabolism, foreshadowing the development of biochemical genetics and molecular biology, and this adds to our knowledge base.^{14–16} This supporting knowledge of individual pathways, control, and stoichiometric constraint has led to the unprecedented role of central metabolism in systems-based studies of energy flow using flux-balance analysis.¹⁷ Finally, because tumors have large metabolic demands, there are associations of the central metabolic genes with cancers.^{18,19} The observation of increased glycolysis in tumors, often called the Warburg effect,²⁰ has led to many proposals using therapeutic approaches targeted to these genes and proteins.^{21,22}

Recently Marden² provided a comprehensive review that raises the question about a pattern that has emerged from a wide variety of studies—that central metabolic enzymes have a disproportionate representation as genes under apparent selection. He emphasizes a growing list of metabolic genes that have so-called pleiotropic “moonlighting” activities, outside the normal functions typically associated with their classic roles as so-called housekeeping genes.^{23–27} For example, hexokinase has been called a “jack of all trades” because of its repeated appearance in novel functions in both plants and animals.²⁸ Finally, Marden² introduces the theme that many of the molecules of metabolism are messengers that signal and integrate other physiological responses with potential fitness consequences. That is an argument that I will emphasize here.

Flux, fitness, and concentration control

The issue of pathway flux and its control has long dominated considerations of the potential for functionally based genetic variation in enzymes to impart fitness variation.^{29–31} This attention derives from the classic 1981 paper by Kacser and Burns³² that introduced the elements of metabolic control analysis³³ into the decades-old question about the causes of genetic dominance.³⁴ The fundamental observation of the theory is that in linear pathways flux control is shared by all the enzymes in the pathway, and as a result each individual step will possess little potential to impart a change in net flux, except at very low activity levels (Fig. 1). This prediction arises from the hyperbolic relationship between activity and flux, where, among single members of a pathway, modest reductions in enzyme activity are expected to only result in biologically insignificant drops in pathway flux. Assuming a corresponding fitness function that equates maximum fitness with maximum flux, there would appear to be little room for modest activity polymorphisms to result in biologically significant fitness effects.³⁰ It has been further pointed out that, assuming this hyperbolic fitness–activity function, as fitness continues to increase by mutations enhancing activity, there is a limit where the diminishing returns from the small incremental fitness increases are countered by the noise of genetic drift. At this point, we have moved into the zone of nearly neutral evolution, and fitness-driven activity increases will stall.³⁵ For this reason, we also expect that enzyme activity for each gene has generally evolved under directional selection to a zone where we expect little effective fitness variation in natural polymorphisms.

The argument of an equal distribution of flux control in pathways challenges the long-held classic notion that specific non-equilibrium enzymes, such as hexokinase or phosphoglucokinase, are rate-limiting steps³⁶ in a pathway and should emerge as the best targets for genetically shifting flux in response to natural selection. While this classical biochemical designation of rate-limiting control is passé, it nonetheless still places the understanding of the pathway distribution of selection responses on identifying those steps with significant flux control and associating the general enzyme and pathway properties that apparently favor the emergence of flux control at these points in any pathway. It might be expected that these properties would correlate with empirical observations on gene-specific molecular variation.

Predictions about the effect of flux control on levels of molecular polymorphism within populations and molecular divergence between species are complex. Other than mutations of large functional loss, more subtle mutations in enzyme steps with no control have no effect on fitness, and on the average will evolve in a fashion consistent with neutral theory. For enzymes with significant flux control, mutations, either as protein changes or expression changes, have an effect on flux and fitness. How this is expressed at the molecular level depends on how flux variation relates to fitness. Under purifying selection for maintaining constant flux, an enzyme with high control will remain conserved and show relatively less interspecific divergence and reduced intraspecific polymorphism than neutral variation. Alternatively, under a scenario of diversifying selection for varying flux in different temporal and spatial environments, these high-control enzymes will see relatively more adaptive fixation and polymorphism than the neutral case. The problem is defining the neutral case. This is usually attacked by the application of tests based on predictions of the neutral theory, such as the dN/dS ratio,³⁷ the HKA test,³⁸ and in particular the McDonald–Kreitman (MK) test.³⁹ In individual cases, statistical rejection of these classic tests discovers genes of interest. Despite this complexity, if one could provide flux control estimates across all the genes in a pathway, then that is an important step towards understanding the cause of different patterns of molecular variation across these genes and their pathways.

Aside from gene-specific flux control, a related question is how metabolite concentrations, namely the substrates, products, and cofactors of individual reactions in a pathway, change under genetic perturbation. In the face of activity perturbation, two outcomes are possible: flux can shift or substrate–products levels can change, or both. The generally universal observation of genetic dominance implies conservation of flux. One simple mechanistic explanation for the general observation of genetic dominance and flux homeostasis is the hypothesis that there is a trade-off between enzyme activity and substrate–product concentrations, and that trade-off maintains flux in the face of genetic perturbation. Under this argument, as enzyme activity is reduced, substrate concentrations, in particular those upstream of the reduced step, increase in concentration, while those downstream products decrease. This thermodynamically drives a higher reaction rate and maintains metabolic flux as an inevitable outcome of first-order kinetics. However, Kacser and Burns³² rejected this explanation and suggested, along the same arguments used to predict flux control, that the substrate–product concentrations in a pathway are also insensitive to genetic perturbation in individual steps. Unfortunately, there is surprisingly little empirical data relating to this the notion that concentration control coefficients (analogous to flux control coefficients) are small or that metabolite concentrations also exhibit genetic dominance. Recently, Fell⁴⁰ revisited this question of metabolite concentration control and emerged with a different conclusion; one that is central to the unfolding arguments in this review. He addresses the question from the stand point of a co-response coefficient that defines the relative impact of a change in enzyme activity on both a particular flux and metabolite concentration. His predictions are that “a change in activity of a single enzyme will most likely produce a larger signal in the metabolite concentrations than in the metabolite fluxes.” Moreover, the concentration co-responses can be quite large and are magnified when the enzyme itself has a large flux control. The importance of this prediction is that it shifts the emphasis from

studying flux-based phenotypes to studying the phenotypes of shifting metabolite concentration and its fitness consequences.

Unfortunately, the evidence that local metabolite levels respond significantly to local genetic perturbation is limited. A recent study in yeast by Fendt *et al.*⁴¹ used several available approaches to explore the effect of reduction of gene function on metabolite levels. They first modulated a set of target genes in yeast through deletion of a common transcription factor (*GCR2*) that is known to perturb expression in set of metabolically central genes. They also shifted individual expression in four central glycolytic genes using strains where the native promoter had been replaced by a Tet-controlled promoter that is repressed by the addition of doxycycline. They simultaneously assayed metabolite profiles down the pathway. Their study clearly shows a trade-off because there were notable increases in upstream metabolite pools, and these were local to the target enzyme. Addressing the same question, Ewald *et al.*⁴² used single-gene knockouts in yeast and again found measurable metabolite increases that were local and upstream to the affected gene. However, the relationship between this type of trade-off and the levels of genetic perturbation associated with polymorphism in nature is unclear. This is because the first class of perturbations affects the global regulation of a number of genes in a functionally associated transcription set, while the second set of single-gene tests are carried out at doxycycline concentrations most likely to produce single knockdowns that are more severe than the variation seen in nature.⁴³ Perhaps in both studies this is an effect of knockdown to near blockage of the pathway. While these studies are informative, we still have no answer to the question of whether metabolite levels show genetic dominance when the associated enzymes steps are reduced by more modest levels than those apparently used in these experiments. Like the attention to flux control, we clearly need many more studies focused on evaluating metabolite concentration control or the corresponding co-response coefficients.

In conclusion, some elevated metabolites may have pleiotropic negative fitness effects,⁴⁴ but others with more benign effects potentially provide important intracellular signals. The sensitivity of metabolite concentrations to genetic perturbation must be explored in greater resolution, a problem that is tractable with modern metabolomics methods. As proposed by Marden,² it is perhaps time to deemphasize flux variation as the principal phenotype under selection.³⁰ While flux variation should continue as a phenotype of interest, especially for polymorphisms that set the levels of energy storage pools of trehalose, triglycerides, and glycogen, I propose that more attention be directed at metabolite-associated phenotypes that are associated with setting overall energy economy. Similar to the arguments about the distribution of flux control, the pathway distribution of metabolite co-response or concentration control will likely depend on properties of the metabolites, enzymes, and pathway architecture.

Pathway architecture

From the early allozyme era, hypotheses based on differences in enzyme class properties and contexts have been offered to explain gene variation in levels of polymorphism.^{45–48} Since central metabolism is universal and conserved, a goal of evolutionary genetics should be to build our understanding of selection on metabolic genes based upon the unique and common

characteristics of each pathway step and shared network properties.⁴⁹ This goal emerges from the proposal that features such as pathway position determine flux control and favor a response to selection at that step. Furthermore, the selection that is associated with the control of apportioning flux at branches will also cause some genes to become the best targets of diversifying selection in a changing environment or targets of positive selection for adaptive fixation. The growth in “omics” tools and systems-based characterizations of the network properties and context of metabolic genes contributes toward this goal.

There has been recent theoretical attention to this question of the context-dependent evolution of flux control and the resulting molecular consequences. Wright and Rausher⁵⁰ first addressed whether, if under selection to increase flux in a *de novo* linear pathway, the greatest flux control would emerge at the head of a pathway and consequently how this emergence would affect patterns of between-species divergence and population polymorphism across all members of the pathway. Their model started with a linear pathway of 2–10 steps that was under selection to increase flux. Random mutations with different function were introduced and subjected to selection to increase pathway flux, and flux control at each step was estimated. Fixation of alleles and levels of contemporary polymorphism were recorded throughout the process. They found that for a pathway that has reached the final fitness-flux optimum, the upstream enzymes tend to have evolved higher control coefficients. At this point, the system is under purifying selection that reduces segregating polymorphism and adaptive fixation at the controlling enzyme at the head of the pathway. Downstream enzymes with low flux control accumulate variation as neutral and slightly neutral alleles. However, in the transition period of evolving towards this higher flux and fitness, the upstream position has higher rates of adaptive fixation. Sellis and Longo⁵¹ followed a similar approach, assuming flux to equate with fitness and that flux control was already vested in the top of a pathway. Their predictions on the emergent patterns of molecular evolution were similar to those of the former authors and were also dependent on the extent to which the evolving system reached the fitness-flux end point. Neither study tells us specifically anything about the appearance of adaptively maintained polymorphism, because their fitness function assumes just purifying selection for increasing flux. It would be interesting to look at the same properties in system where the fitness-flux function has several shifting optima and would more likely lead to polymorphism.

These studies both emphasize linear pathways, while metabolic systems are often reversible and highly branched, and both are expected to impact flux control.⁵² Rausher⁵³ explicitly addressed the branching question, building upon the simulation approach used in Wright and Rausher.⁵⁰ He contrasted two models, assuming either subsaturation or saturation kinetics, and concludes in both cases that flux control evolves to be concentrated in the enzymes at branch points. Classes of mutational change (advantageous substitutions, transient polymorphism, and deleterious polymorphism and fixation) follow the earlier predictions of flux control, and as in the former studies depend on the state of the system with respect to distance from the fitness-flux optimum. However, in a system where flux is continuing to shift over the appropriate time scale, one might expect there to be excess adaptive fixations at branch points. Overall, this validates the informal predictions made in Flowers *et al.*⁵⁴

Most empirical data addressing molecular evolution in pathways is not the result of motivation to understand the selection to maintain polymorphism but on how functional constraint is manifest between species as reduced amino acid substitution across pathways and among genes. While not targeted to polymorphism, these studies still offer some insight on the question of control distribution and constraint. Focusing on genes of metabolism in yeast, Vitkup *et al.*⁵⁵ showed a significant relationship between implied evolutionary constraint (or the rate of molecular evolution) and enzyme network connectivity. Their logical argument is that the most highly connected steps have the greatest pleiotropic effects and will be the most constrained. They observed that the central and the most highly connected enzymes do evolve more slowly, as well as those enzymes bearing high metabolic flux. This distribution-of-constraint argument has been the philosophy behind not only central metabolism, but also other network-based studies, such as those associated with yeast;⁵⁶ the anthocyanin, terpenoid and the gibberellin pathways in plants;^{57–59} the immune systems in *Drosophila*;⁶⁰ and the elements of the fundamental metabolic signaling pathways in *Drosophila*.^{61,62} Most of these studies have not focused on the role of pathway structure or properties on intraspecific variation or polymorphism, but instead on relative selective constraint.^{63,64}

In a novel study, Colombo *et al.*⁶⁵ used measures of direct metabolic flux in the red blood cell and compared this flux to molecular divergence across 35 great ape orthologues. This study can be singled out as special because the red blood cell is a well-modeled system that serves as a detailed classic model of flux distribution.^{66–68} The analysis concluded that enzymes supporting higher flux, in both stressed and non-stressed states, had lower interspecific divergence. Interestingly, network features reflecting centrality, such as “closeness” and “betweenness,” showed no significant correlation with evolutionary divergence.

Whitt *et al.*⁶⁹ studied both divergence and polymorphism in the six genes of the maize starch pathway in 30 inbred lines of maize. The application of the neutrality-based HKA test³⁸ to these genes found significantly lower polymorphism relative to divergence in two genes, thus rejecting the hypothesis that these genes are evolving a neutral fashion. This departure is considered to be the outcome of maize domestication and artificial selection. It is not particularly clear why these two particular genes responded, although one enzyme, *bt2*, the small subunit of ADP-pyrophosphorylase, is considered to perform a rate-limiting step, and the other is involved in partitioning ADP-glucose between the pools of amylose and amylopectin.

While not focused on central metabolism per se, Olson-Manning *et al.*⁷⁰ studied, in *Arabidopsis thaliana*, the relationship of flux control and position in the glucosinolate pathway and included estimates of polymorphism and divergence for each step. The pathway core consists of seven steps. Flux was quantified for each of the seven glucosinolates in the pathway, and flux control for each enzyme was estimated by the heterozygous knockdown using transfer DNA (T-DNA) insertions. The initial enzyme in the pathway, CYP79F1, was clearly demonstrated to possess significant flux control. The DNA sequence data on both intraspecific and interspecific variation (to *A. lyrata*) produced significant MK tests³⁹ for both CYP79F1 and SUR1 (which is farther down the pathway).

This widely used neutrality test contrasts, in a two-by-two table, the ratio of the number of fixed synonymous mutations between two species and the number of synonymous polymorphisms (both assumed to be neutral) with the respective numbers of non-synonymous replacement or amino acid mutations. This is tested by a simple contingency G -test. In both cases, the test rejection was due to excesses of non-synonymous polymorphisms, which is consistent with the action of diversifying selection favoring amino acid polymorphism. This study supports the contention of Wright and Rausher⁵⁰ that flux control will appear at the beginning of linear pathways, as will adaptive polymorphisms if there is diversifying or balancing selection for changing glucosinolate levels. Since glucosinolates are important defensive compounds against herbivores, the connection to fitness is also apparent.

On the basis of arguments that branching pathways present special opportunities for adaptive selection targeted at branch-point enzymes, Flowers *et al.*⁵⁴ studied molecular evolution along the glycolytic pathway. The study explored both intra- and interspecific sequence variation in 17 genes in *D. melanogaster* and *D. simulans*. Some of these enzymes converge at glucose-6-phosphate, the point where partitioning to various storage pools overlaps. The case-by-case rejections of MK tests concentrate primarily on enzymes at the top of the pathway, at glucose-6-phosphate, suggesting that selection is acting on the partitioning to the storage pools of glycogen and trehalose and the pentose shunt. It is worth noting that, at the bottom of the pathway, after splitting into trioses and where flux must double, the pathway is much more conserved. It should also be added that SNPs in many of the genes, particularly those genes at the top of the pathway, show latitudinal SNP clines in *D. melanogaster*, suggesting geographic selection targeted at this pathway junction.⁷¹

Another question associated with pathway architecture and selection is the coordination of modular responses, where features, in particular gene-specific expression, are coordinated in response to shifting external environments and internal shifts in demand. There are many dozens of studies using microarrays to discover differential gene expression shifts under stress and nutritional challenge.^{72–74} These studies clearly demonstrate modular physiological responses of transcription to dietary changes. This observation is important because metabolic control–analysis theory predicts, because of the insensitivity to single-step perturbations of flux, that large physiological responses must involve multistep coordination of enzyme capacity changes to affect large changes in flux.^{75,76} The interesting question is whether the adaptive genetic responses to stress operate along the same axis and direction and have the same modularity as the transcriptional shifts in the induced modules to the same stress. Selection either from the standpoint of constraint or polymorphism must be studied in the context of pathway architecture and expression modules.

Energy-state signaling

If we wish to focus on levels of intermediary metabolites as a primary phenotype of metabolic pathways, we need to consider how metabolite levels, acting as the energy-state readout of current metabolism, translate into larger organism-level responses that determine energy utilization and contribute to fitness variation. Overall, organismal metabolism is not just a series of anabolic and catabolic reactions, but involves physiological shifts in the

utilization of energy during the individual's life span. We assume that this process is fine-tuned, such that any deviation from the optimal allocation of energy in particular environments has fitness costs. To regulate this process, all organisms possess signaling pathways in specialized cells to translate the metabolic condition of the individual and promote transcriptional decisions that affect energy utilization and storage. Over the life span of an individual, this changing utilization affects fitness-related phenotypes, such as fecundity schedules and trade-offs with life span.^{77–79} There are a number of well-characterized and widely reviewed signaling systems.^{80,81} These signaling systems are interconnected and share the common feature of responding to both intermediary metabolites and adenylate charge.⁸² As such, any general signaling system is expected to be intimately coupled at the top with the central metabolic pathways and their perturbations on these metabolites.⁸³ These signaling systems also have direct medical connections because of their relationships to both human longevity research and metabolic disease.⁸⁰ Figure 2 depicts such a generalized sensing system, and I briefly discuss three systems below.

A well-studied insulin-like signaling pathway plays a conserved role in metabolic homeostasis and affects life span in *Drosophila* and *C. elegans*.^{84–86} In many ways, this system recapitulates the well-studied features of β -pancreatic insulin signaling in mammals.^{87,88} In that system, while glucose from the blood and its subsequent metabolism generates metabolite signals, it is the ATP:ADP ratio in the mitochondria that opens K_{ATP} channels in the outer membrane, depolarizes the plasma membrane, transports Ca^{2+} through voltage-gated Ca^{2+} channels, and results in vacuoles undergoing exocytosis and releasing insulin into the blood stream. The mitochondria plays a key role because it is the efflux of intracellular metabolic intermediates (ATP, GTP, cAMP, NADPH, glutamate, malonyl-CoA) from the metabolism of pyruvate that couple to and shift the ATP:ADP ratio. It is the metabolism of ATP from both Krebs-derived and shuttle-derived NAD/NADH that couples these cofactor intermediates to signaling. Similar to the pancreas, there are neurosecretory cells in *Drosophila* that produce and release hormones and insulin-like peptides (ILPs), presumably in response to energy-state molecules.⁸⁹

In humans, this signaling response in the β -pancreatic cells can be disturbed by mutations in enzymes that affect the levels of coupling metabolic intermediates. The most dramatic and best studied are mutations in human glucokinase⁹⁰ and mitochondrial-expressed glutamate dehydrogenase that result in diabetes and hyperinsulinism.⁹¹ Furthermore, there are a wealth of experimental studies from mammalian cell lines showing that perturbation of metabolic enzymes and the mitochondrial carriers disrupts insulin release.^{92–94} The role of the levels of AMP, ADP, ATP, and other metabolic intermediates in insulin release ties genetic variation in metabolic enzymes to potential variation in signaling responses to changing nutritional conditions.

In insects, a system analogous to the glucagon system in mammals involves adipokinetic hormone (AKH), which is released under starvation and leads to the mobilization of glycogen.^{95–97} Ablation of special neurosecretory cells for AKH⁸⁹ significantly affect glycogen and trehalose storage.^{98,99} Moreover, the mechanism of energy-state signaling that leads to AKH secretion appears to be associated with AMP-activated protein kinase (AMPK) sensing, as discussed below.¹⁰⁰

A third well-studied universal signaling system involves AMPK. This system directly responds to the AMP:ATP ratio or the adenylate energy charge.^{101,102} When this ratio is shifted by energy imbalance, as under starvation, increasing AMP levels result in activation of AMPK by phosphorylation. Activated AMPK in turn phosphorylates important downstream enzymes and leads to the inhibition of energy consumption. For this role, AMPK has been termed the “fuel gauge” of metabolism.¹⁰³ This sensing mechanism has been associated with coupling metabolic cycles with circadian clockwork cycles,¹⁰⁴ as well as starvation-induced hyperactivity in flies.¹⁰⁵ Recently, Braco *et al.*¹⁰⁰ knocked down AMPK in the AKH-producing cells of *D. melanogaster* and observed disruption of AKH secretion. Likewise, transgenic overexpression of AMPK in adult *Drosophila* fat body and muscle extended life span (mimicking starvation). Conversely, knockdown with RNAi reduced life span, and these effects were modified by supplementing with adenosine.¹⁰⁶ In the mouse skeletal muscle, AMPK also increases NAD⁺ cellular levels, thus enhancing the sirtuins that modulate the transcription of downstream metabolic genes.¹⁰⁷ It should be emphasized that, while Figure 1 depicts a system with a top-down specialized cell and hormone-based signal, sensing and the downstream responses can also take place within other tissues independent of this signal.

It must be emphasized that the common feature of all these energy sensory systems is that signaling responses depend directly or indirectly on adenylate charge. These extend not only to AMP, ADP, and ATP, but to the cofactors NADH and NAD, and to NADP and NADPH, as the readout of immediate cellular energy state. Aside from the interconversion with NADH, NAD plays numerous further roles, such as protein modification through ADP-ribosylation reactions that are associated with NAD consumption. In addition to the downstream effects of the signaling systems above, there is growing evidence that NAD and NADH can directly influence gene transcription.¹⁰⁸ There is also overwhelming evidence that the effects of dietary restriction are embedded in pathways that directly involve NAD-associated mechanisms in several ways.^{109–111} These mechanisms more likely involve the sirtuins as NAD-dependent protein deacetylases or ADP-ribosyltransferases that have direct effects on transcription.¹¹²

The involvement of NAD/NADH and these coupling metabolites directly implicates the mitochondria in nutrient signaling and aging.^{113–117} In our study of clinal variation in *D. melanogaster*,⁷¹ *Gdh* (glutamate dehydrogenase; GDH) emerged as a major factor in expression variation that is predicted to increase in southern populations. Mitochondrial NAD-dependent GDH, glutamate, and α -ketoglutarate are considered major targets in energy sensing^{118,119} and in insulin secretion.^{91,120} The facts that glutamate is one of the most abundant metabolites, sits at the epicenter of the metabolic pathways, and is a highly connected metabolite may contribute to this important signaling role.¹²¹ In our study of life span extension in *Drosophila* using engineered genotypes,¹²² we demonstrated that a modest genetic reduction of GDH of only 30% strongly extended life span, and this extension was magnified under dietary restriction.

The metabolite signaling associated with the central metabolic genes goes beyond sensing nutrient inputs. In his review, Marden² discusses hypoxia signaling and its coupling to Krebs metabolism. The hypoxia-inducible pathway and the hypoxia-inducible factor HIF-1 α

protein in effect sense oxygen level via the TCA pathway. The HIF-1 α protein is constitutively expresses in most tissues, but is rapidly degraded in the presence of normal oxygen levels. As TCA cycle intermediates (α -ketoglutarate and succinate) accumulate under hypoxia, these inhibit the enzymes that degrade HIF-1 α . The accumulating subunit binds to the HIF-1 β subunit and increases the expression of a number of genes, including those associated with morphological changes for enhancing oxygen delivery. Marden and colleagues have extended this argument to succinate dehydrogenase (SDH) polymorphism and the study of Granville Fritillary metapopulations, where flight performance is believed to be coupled to dispersal ability as a fitness trait, and the SDH polymorphism is proposed to be associated with morphological changes that facilitate oxygen delivery.¹²³ This type of observation adds another level of complexity to the role of sensing through metabolic intermediates of the TCA and mitochondria.

Despite the importance of *D. melanogaster* in studies of life span extension and dietary restriction^{124–127} and the recurring implication of central metabolism in aging, there is little evidence in this species for the effect of reducing expression of the central metabolic genes on life span. To address this missing piece, Talbert *et al.*¹²² used P-element gene-specific knockdown to modestly reduce the expression of seven genes that all involve co-factors and intermediates that couple to sensing (*Hex-A*, *Hex-C*, *Gdh*, *Gpdh*, *Men*, *Idh*, *Mdh2*). The female life spans were measured for both normal and restricted diets and the genotypes used paired P-element excision alleles in otherwise isogenic backgrounds. Dietary restriction (DR) is well established to extend life span in *D. melanogaster*. The results showed that, for five of seven genes, a reduction of activity extended life span on one or both diets; moreover, the effect tends to be amplified under DR. While we cannot directly ascertain the mechanism, only 30–50% reductions in normal expression of these genes often extend life span, and this extension occurs at levels of gene expression reduction that generally bracket natural expression variation. This study has major implications for understanding the fitness effects of functional polymorphism in these genes.

Much research, largely motivated by aging questions, has focused on physiological signal transmission systems that drive downstream responses to energy-input levels and allocate energy usage and storage. It is commonly stated that these systems sense the energy condition, and the focus then is generally on the downstream process, and the top-end mechanism of sensing is passed over. Concerning this top-end, there is a very clear connection between central metabolic pathways and the coupling metabolites that appear to set these sensing systems, and in particular this involves adenylate charge. This connection provides mechanisms for the transmission of metabolite messages that respond to nutrient input. If genetic variation in the core metabolic pathways modifies these intracellular metabolite levels, then these genotypes act as different set points with different fitness effects in different environments.

Metabolite concentrations as phenotypes

As a direct readout of the current energy-state status, the primary metabolite levels of the energy-processing pathways are the proximal signals that begin the cascade of the well-known signaling pathways toward quantitative traits associated with the economy of energy

expenditure. This places functional variation in the enzymes of central metabolism and their potential to shape intracellular metabolite levels as important targets of selection. The growth of high-throughput metabolic profiling is a tool for understanding pathway responses to environmental conditions and stresses.¹²⁸ Some energy-storage pools (such as trehalose and glycogen) show well-known direct responses to environmental challenges, such as thermal stress and desiccation, and as such reflect the apportioning of influx to those pools. However, some intermediary metabolites are also signaling molecules and represent the bridges that connect pathways (see Table 4 in Ref. 2). Thus, genetic variation in metabolic genes may be an important source of functional variation in signaling through its effects on metabolite concentrations, and more attention should be devoted to this genotype–phenotype relationship.

As raised earlier, a tenet in the work by Kacser and Burns³² was that, as pathway flux is unresponsive to genetic perturbation, so are pathway metabolite levels insensitive to genetic perturbation. If so, then there is little reason to suppose that genetic variation in central metabolic genes can act through signaling mechanisms. However, as emphasized earlier, there is reason to think that metabolites, unlike flux, may be very sensitive to single-gene perturbation.⁴⁰ Metabolite levels are observed to possess significant heritability,^{129–132} so the empirical questions at this junction are several. To what extent does discrete genetic perturbation at the modest functional levels seen in nature control metabolite concentrations? How are the concentration control coefficients distributed across a metabolic network? And how sensitive are downstream signaling responses to modest changes in certain metabolite concentrations?

There is a large literature examining the phenotypic coordination of gene expression and metabolite levels.^{133,134} Metabolite and gene expression levels are highly plastic and show predictable and large responses to environmental shifts, such as diet, stress, aging, and the shifting demands of cell replication.^{132,135–142} For example, starvation in *Drosophila* strongly shifts the NAD/NADH ratio through increases in the level of NADH and minimal shifts in NAD. Likewise, it significantly drops the level of NADP, consistent with reduced fatty acid synthesis.¹⁴³

While the levels of some metabolites are robust to external perturbations, network structure and pathway position can affect which metabolites shift in response to perturbation.^{136,144} The modularity of metabolism places some metabolites at the center of pathway clusters.¹³⁶ It is these metabolites, and the genes that affect them, that we should focus on as the targets of selection and those likely to be responsive to genetic perturbations that will affect energy-expenditure phenotypes. One might expect that metabolites central to network structure and possessing high intracellular concentrations are potential signaling molecules that might serve as control hubs.¹²¹ Ideally, this can be explored directly in an experimental context and indirectly through population genetics, as outlined here. Since responses are typically modular, and all features of pathways are not equally responsive, we might expect to see that coordinated patterns of gene expression in the components of a metabolic network will also show coordination of genetic expression changes on geographic or temporal scales.

There are limited opportunities to combine studies of pathway modularity with natural selection. In Lavington *et al.*,⁷¹ we began in *D. melanogaster* to integrate SNP expression variation, pathway modularity, and geographic selection by screening 46 metabolic genes for 127 SNPs. We tested for clinal shifts in SNP alleles across 20 population samples collected across the eastern U.S. latitudinal cline from Maine to Florida. In parallel, the allele-specific *cis*-expression contribution for each clinal SNP was approximated by using the gene expression data from the DGRP panel of 37 lines.¹⁴⁵ Most (65%) of the genes possessed significant clines in at least one SNP. When changes in these SNP alleles were associated with significant allele-specific expression, it is possible to predict how gene expression might change in a coordinated modular fashion with latitude across populations that experience dramatic life-history challenges.^{146,147} A principal component analysis (PCA) of the variance–covariance matrix of predicted expression changes across the cline allowed us to rank genes by their clinal contribution to overall expression and the direction. When these genes were studied in the context of the central metabolic pathway, they suggested important directional changes. Interestingly, the metabolites of pyruvate, α -ketoglutarate, and especially glutamate are associated with high-ranking genes, such as *Idh- β* , *Pdk*, *Got2*, and *Gdh*. Notably, the top of the glycolytic pathway, especially the pentose shunt, showed overall reduced expression with increasing latitude compared to the lower Krebs pathway, which generally shows increased expression with increasing latitude. While this study is necessarily noisy and limited in scope, it precisely points the way for future work. Whole-genome sequencing and experimental RNA-seq will provide a greater number of genes, many more SNPs per gene, and better estimates of *cis*-based gene expression.^{148,149} This new gene expression and sequence data, when integrated across entire pathways, can be interfaced with geographic data for populations where different life-history shifts are associated with dissimilar pressures on energy storage and utilization.

Closing comments

The world is a heterogeneous environment requiring different metabolic signaling responses to meet different stress conditions. It would appear that the most appropriate selection models for many metabolic genes take the form of multiple-niche selection.¹⁵⁰ In this selection model, different genotypes possess different fitnesses in different niches, and overall this is capable of maintaining genetic diversity. When interfaced with genotype-specific energy signaling that is associated with differential survival and fecundity in different environments, this type of selection could explain the presence of genetic variation in many genes of central metabolism. Metabolites respond at short time scales to a myriad of factors, nutrient input quality and quantity, and hormonal shifts, and do so in a modular fashion. On top of these general environmental responses, we posit that genetic variation, whether catalytic or expression based, of the component metabolic enzymes establishes a background of response variation in energy-state signaling for the same nutrient input.

Three decades ago, Brian Clarke¹⁵¹ outlined the series of conditions that he felt must be met to satisfactorily build cases about selection acting on allozyme polymorphism. These conditions were generally associated with dispensing with the problem of linkage association by focusing on the function of the gene or enzyme at hand. The new genomics and sequencing technology, integrated with theoretical population genetics modeling,

systems biology, and computational tools, now go well beyond this condition and allow both bottom-up and top-down approaches to studying adaptive variation.^{152–160}

The highly successful study of conspicuous phenotypic features, such as coat color in *Peromyscus*^{161–163} and skeletal variation in sticklebacks^{164, 165} (see table in Ref. 152), have considerably enriched our understanding of the intensity of selection on phenotypic variation and how it can be translated down to the single-gene level. In these cases, selection coefficients are potentially measurable and typically start with a phenotype showing association with agents of selection (such as predation). The allele effects are usually very large. Genomic mapping leads to candidate genes which often have been on the a priori list of functional associates. These are great success stories, and we are likely to see many such single-gene stories. However, it is still unclear if these cases are representative of much of adaptive variation in nature.¹⁶⁶

In contrast, the study of adaptive polymorphism in metabolic genes is much more complex and presents challenges to the idealized goals of linking genotype to phenotype and phenotype to fitness. However, it is hoped that the emergent observations on these genes and their integration into a larger functional context are more likely to represent generalizable rules, since all organisms possess common metabolic pathways with shared features and properties. Nonetheless, there are significant issues associated with inferring and estimating selection intensities that are likely to be much more subtle than in the above cases of conspicuous phenotypic variation. Assessing single-gene selection intensities will likely remain beyond the power of any feasible experiment, and there are challenges with experimental selection.^{167–169} Building upon understanding selection in a metabolic pathway requires a coupling of observations at a number of levels.^{2,123,170} It requires a clear description of population genetics of the variation that might propose a pattern of historical selection and a decoupling of linked effects. There should be a clear description and understanding of the functional effects of genic variation in either expression or catalytic function. Hopefully, there is coupling of metabolic function with the ecology that is imposing selection and geographic or temporal change that can be logically coupled to the metabolic function and inferred phenotype. Preferably, there needs to be experimental genetic manipulation that recapitulates the phenotypic effect. Ideally, there should be independent complementary reinforcing or corroborating observations.¹⁷¹ Obviously, meeting all these later criteria are best suited to model species, where the full battery of tools can be unleashed, although many of the most fascinating stories will still involve non-model species and their ecology.¹⁶⁰

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Figure 1.

Flux control and the effect in a five step pathway. (A). The classic prediction of the 1981 paper by Kacser and Burns.³² The impact of activity variation at a single step on flux decreases as the number of steps in the pathway increases. Control is distributed equally across all steps. (B) The effect of changes in flux for enzymes B and E with different levels of flux control. At upper levels of activity, the effect on flux is notably weaker for enzyme E. For enzyme B, genetic variation in activity will translate into much larger variation in flux and fitness.

Figure 2.

Generic example of energy-state sensing. This is composite generalization of the features of a hormonal-based signaling system. It has features borrowed from β cell pancreatic insulin and *Drosophila* AKH signaling. Other energy-sensing systems differ from this model.