The Journal of Experimental Biology 214, 179-190

doi:10.1242/jeb.046912

Microevolution of intermediary metabolism: evolutionary genetics meets metabolic biochemistry

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Accepted 30 July 2010

Summary

During the past decade, microevolution of intermediary metabolism has become an important new research focus at the interface between metabolic biochemistry and evolutionary genetics. Increasing recognition of the importance of integrative studies in evolutionary analysis, the rising interest in 'evolutionary systems biology', and the development of various 'omics' technologies have all contributed significantly to this developing interface. The present review primarily focuses on five prominent areas of recent research on pathway microevolution: lipid metabolism and life-history evolution; the electron transport system, hybrid breakdown and speciation; glycolysis, alcohol metabolism and population adaptation in *Drosophila*; chemostat selection in microorganisms; and anthocyanin pigment biosynthesis and flower color evolution. Some of these studies have provided a new perspective on important evolutionary topics that have not been investigated extensively from a biochemical perspective (hybrid breakdown, parallel evolution). Other studies have provided new data that augment previous biochemical information, resulting in a deeper understanding of evolutionary mechanisms (allozymes and biochemical adaptation to climate, life-history evolution, flower pigments and the genetics of adaptation). Finally, other studies have provided new insights into how the function or position of an enzyme in a pathway influences its evolutionary dynamics, in addition to providing powerful experimental models for investigations of network evolution. Microevolutionary studies of metabolic pathways will undoubtedly become increasingly important in the future because of the central importance of intermediary metabolism in organismal fitness, the wealth of biochemical data being provided by various omics technologies, and the increasing influence of integrative and systems perspectives in biology.

Key words: evolution, life history, lipid metabolism, electron transport, anthocyanin, enzyme polymorphism, metabolic control analysis, chemostat selection.

Introduction

Initial evolutionary studies of biochemical function, which gave rise to the field of comparative biochemistry, focused primarily on species-specific characteristics or inter-specific differences in biochemical traits, most notably aspects of enzyme function and metabolism (Somero, 1969; Baldwin, 1970; Hochachka, 1973; Hochachka and Somero, 2002). These investigations were mainly undertaken by biochemists or physiologists, were heavy on biochemistry but light on evolution, and were published mainly in physiological or biochemical journals. Starting in the late 1960s to 1970s, evolutionary biologists began using genetically variable enzymes (allozymes, enzyme polymorphism), as convenient experimental models to investigate general evolutionary-genetic issues, such as the relative importance of natural selection *versus* random genetic drift in micoevolution (short-term evolution; population genetics). Initial studies were heavy on evolution but light on biochemistry, and were mainly published in evolutionary or genetics journals (Lewontin, 1974; Selander, 1976) (reviewed by Mitton, 1997). However, detailed knowledge of enzyme function was increasingly viewed as being essential to adequately distinguish among competing evolutionary hypotheses (e.g. neutralist–selectionist debate), and in-depth functional characterizations of enzymes progressively became an important research focus (Koehn et al., 1983; Eanes, 1999; Watt and Dean, 2000). This development initiated a deeper, more balanced synthesis between functional biochemistry and microevolution (population genetics), involving both sophisticated biochemical approaches [e.g. kinetic analyses of catalytic efficiency (Hall and Koehn, 1983)] and powerful population-genetic analyses of enzyme evolution (Eanes, 1999; Storz and Wheat, 2010).

During the past decade, microevolutionary studies of biochemical function have expanded to focus more and more on adaptive changes in pathways of intermediary metabolism as opposed to functional aspects of an individual enzyme. For example, as discussed in more detail below, microevolutionary studies of intermediary metabolism have investigated, from a fresh perspective, long-standing issues in evolution that have not previously been studied from a biochemical viewpoint [hybrid breakdown and speciation, parallel evolution (Burton, 2006; Rausher, 2008; Streisfeld and Rausher, 2009)]. In addition, metabolic information has expanded our understanding of traditional topics of biochemical evolution such as biochemical adaptation to temperature (Eanes, 1999), as well as contributing significantly to basic studies of the genetics of adaptive evolution (Rausher, 2008), and the evolution of complex traits, such as life history (Zera and Harshman, 2009; Zera and Harshman, 2011). Finally, microevolutionary studies of intermediary metabolism are contributing significantly to the development of evolutionary systems biology, which focuses on the evolution of whole networks (e.g. glycolysis) (Eanes, 1999; Eanes, 2011), and the effect of network attributes on the evolution of individual proteins (e.g. 'pathway position' and rate of protein evolution) (Eanes, 1999; Dykhuizen and Dean, 2009; Rausher et al., 1999; Rausher et al., 2008; Wright and Rausher, 2010).

Why the increased focus on the evolution of pathways of metabolism?

A number of factors have resulted in an increased interest in the evolution of intermediary metabolism, most notably: (1) increasing appreciation of the importance of integrative, multi-level investigations; (2) an increasing shift to 'network' or 'systems' thinking; and (3) recent technological developments (omics) that allow multiple components of pathways to be characterized simultaneously. Since the 1980s there has been an increasing focus in evolutionary biology on integrative studies that link investigations across multiple levels of the biological hierarchy, often from the gene to whole organism (Koehn et al., 1983; Feder and Watt, 1992; Eanes, 1999; Rausher, 2008; Zera and Harshman, 2009) (Fig. 1). In these studies, intermediary metabolism often occupies a central 'gateway' position, providing the link between molecular variation and variation in whole-organism performance.

One type of integrative study can be classified as 'bottom up', in which the goal is to understand the extent to which molecular or biochemical variation alters fitness and hence can be acted on by natural selection. A classic example of such a 'vertical, bottom-up' type of study is the investigation of enzyme polymorphism (Koehn et al., 1983; Watt, 1991; Eanes, 1999; Watt and Dean, 2000; Storz and Wheat, 2010). It has long been recognized that biochemical differences among allozymes (genetic variants of a particular enzyme) are necessary but not sufficient to affect organismal fitness. To affect fitness, allozymes must differentially influence flux through the pathway in which they function. However, given the often non-linear, hyperbolic relationship between enzyme activity and pathway flux (Kacser and Burns, 1979; Kacser and Burns, 1981; Dykuizen and Dean, 1990), in which a large change in enzyme activity often produces only a small change in flux, such allozyme-dependent modulation of flux is by no means certain. This realization brought investigation of allozymes and pathway flux to the forefront in evolutionary studies of allozyme function, and this refocusing on pathways of metabolism has continued up to the present day (Koehn et al., 1983; Eanes, 1999; Eanes, 2011; Dykhuizen and Dean, 2009).

In other cases, the motivation for an integrative investigation has been to understand the evolution of a whole-organism phenotype, by understanding how the underlying components of the trait evolve. This type of study can be classified as 'top down' (Zera and Harshman, 2009; Zera and Harshman, 2011; Dykhuizen and Dean, 2009). For example, as discussed in more detail below, to understand the evolution of flower color, or a life-history trait such as dispersal, workers have investigated changes in (i) the pathways of intermediary metabolism that produce flower pigments/lipid flight fuel, (ii) the enzymes that comprise the pathways, and (iii) the genes that encode the enzymes (Rausher, 2008; Zera and Harshman, 2009). Moreover, because these pathways consist of many interacting components (e.g. enzymes), these studies often involve 'horizontal' as well as 'vertical' investigations (Fig. 1). Whatever the motivation, investigation of the causes and consequences of modifications of intermediary metabolism plays an important role in these integrative studies of adaptation.

In addition to the rise in importance of integrative studies, there has been an increasing realization of the importance of network or systems thinking in evolutionary analysis (Eanes, 1999; Dykhuizen and Dean, 1990; Dykhuizen and Dean, 2009; Rausher et al., 2008). Many adaptations consist of a network of interacting components, and to understand how such an adaptation evolves, it is necessary to understand how the component traits evolve through their influence on some systemic property. Conversely, the functional position of a component in the network can strongly influence the evolutionary dynamics of that component. Pathways of intermediary metabolism are prime examples of networks and there has been increasing interest in the evolution of systemic properties of metabolic pathways [e.g. the evolutionary changes in glycolytic flux (Eanes et al., 2006; Eanes, 2011)], as well as the influence of pathway properties on the evolution of pathway components [e.g. rate of evolution of an enzyme can be dependent upon its pathway position; e.g. branchpoint *versus* downstream (Eanes, 1999; Rausher et al., 1999; Rausher et al., 2008; Wright and Rausher, 2010)]. This increasing focus on systemic properties is also bringing pathways of intermediary metabolism to the forefront of studies of evolutionary biochemistry.

Finally, the development of various omics approaches (genomics, transcriptomics, proteomics) has produced an unprecedented wealth of information on variation in components of intermediary metabolism. This information, in turn, has provided the opportunity to investigate evolutionary changes in whole pathways, which has made an important contribution to the increased interest in adaptive changes in pathways of metabolism (Frera et al., 1999; Gracey and Cossins, 2003; van Strallen and Roelofs, 2006; Larracuente et al., 2007; Greenburg et al., 2008; Matzkin and Markow, 2009; St-Cyr et al., 2008; Eanes, 2011). In the following section, I focus on five prominent areas of recent research on the microevolution of intermediary metabolism.

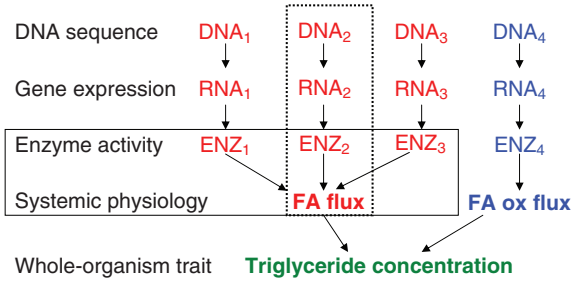

Fig. 1. Diagram illustrating vertical and horizontal integrative studies of the functional causes of adaptation as applied to components of life-history variation in *Gryllus*. Vertical studies (within the dotted lines) focus on the chain of causality of a single factor through several levels of the biological hierarchy. In this case, variation in the sequence and/or expression of a particular gene results in associated variation in enzyme activity, flux through the pathway in which the enzyme functions, and, finally, variation in a whole-organism trait. Horizontal studies (within the solid lines) focus on multiple components of variation at a particular level of the biological hierarchy. In this case, variation in multiple enzymes gives rise to variation in flux through the pathway in which the enzymes function. Subscripts refer to different genes, products of the genes, etc. In this example, the focal aspects of systemic (whole-organism) physiology are fluxes through the pathways of fatty-acid biosynthesis (FA flux) and fatty-acid oxidation (FA ox flux), which contribute to whole-organism triglyceride concentration.

Major research foci

Life-history evolution, laboratory selection and intermediary metabolism

For decades, life-history evolution has been a prominent focus of evolutionary research (Roff, 2002; Stearns, 1992). The overall goal has been to understand why and how species, populations, or genotypes differ in key life cycle attributes, such as the relative duration of juvenile and adult stages, longevity, timing and amount of reproductive effort. A prominent issue has been the functional causes of differences in individual life-history traits, and trade-offs

between traits, such as the commonly observed negative association between reproductive effort and longevity or various aspects of somatic function (e.g. dispersal ability) (Townsend and Calow, 1981; Zera and Harshman, 2001; Zera and Harshman, 2009). Because life-history traits are genetically complex, various quantitative-genetic approaches, such as laboratory selection, have been used to investigate their functional underpinnings [for extensive discussion of this topic see Zera and Harshman (Zera and Harshman, 2009)]. Genetic stocks, selected for divergent life-history traits in the laboratory, have been characterized for a variety of physiological and/or biochemical traits to identify the functional-genetic causes of the evolved differences in life history.

Because modification of organismal energetics has long been thought to drive much of life-history evolution, various aspects of metabolism have been central foci in functional studies of life-history evolution (Zera and Harshman, 2001; Zera and Harshman, 2009; Harshman and Zera, 2007). One prominent aspect of this topic has been alteration of somatic energy reserves (reviewed by Zera and Harshman, 2001; Zera and Harshman, 2009). Lines selected for enhanced somatic functions (extended longevity, starvation or stress resistance, dispersal ability) often exhibit reduced reproductive effort and fecundity and *vice versa*. Enhanced somatic function is often associated with elevated somatic energy reserves, most notably lipids or glycogen, whereas increased reproductive effort is often associated with reduced levels of reserves. The existence of these correlations has led to the idea that evolutionary changes in life history require changes in the relative allocation of an internal resource pool. Importantly, a change in relative allocation is not only required to increase a particular life-history function but this also decreases allocation to other functions, which gives rise to trade-offs. Despite the strong impact of this theory on life-history studies, only recently have detailed metabolic studies been undertaken to identify the specific pathways of intermediary metabolism that have been altered (Zera and Harshman, 2001; Zera and Harshman, 2009; Harshman and Zera, 2007).

Biochemical studies of lipid metabolism

Lipid metabolism has been an intensively studied aspect of intermediary metabolism with regard to life-history microevolution. This is particularly the case for lines of *Drosophila melanogaster* that have been selected for differences in longevity, starvation and stress resistance, and lines of the wing-polymorphic cricket *Gryllus firmus* producing either a flight-capable morph [LW(f): long-winged with functional flight muscles] with low reproductive effort, or a flightless morph (SW: short-winged, with underdeveloped, non-functional flight muscles) with enhanced egg production (Zera and Harshman, 2001; Zera and Harshman, 2009). In the case of *Gryllus*, the polymorphism is found in natural populations, and lipid is the major flight fuel used by the dispersing LW(f) morph. Also relevant are studies of lines of *Mus musculus* that have been directly selected for increased or decreased fat content, and which also differ in life-history characteristics (Hastings and Hill, 1990; Asante et al., 1989).

In *Drosophila*, *Mus* and *Gryllus*, activities of enzymes involved in the *de novo* pathway of fatty acid biosynthesis (ATP-citrate lyase, fatty-acid synthase) and/or enzymes involved in the production of NADPH required for reductive fatty acid biosynthesis (malic enzyme, $NADP^+$-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase), are elevated in genetic stocks with elevated lipid reserves (Hastings and Hill, 1990; Asante et al., 1989; Harshman and Schmidt, 1998; Zera and Zhao, 2003). In the more extensive *G. firmus* and *M. musculus* studies, activities of essentially all lipogenic enzymes are elevated to approximately the same degree. Studies in both *Gryllus* and *Mus*, employing a variety of radiotracers (in the form of H_2O, citrate, acetate or fatty acid) have demonstrated that elevated lipogenic enzyme activity is genetically correlated with elevated *in vivo* flux through the *de novo* pathway of fatty acid biosynthesis (Zhao and Zera, 2002; Asante et al., 1991) (Fig. 2). These studies demonstrate a remarkable global genetic modulation of the enzymes that alter flux through the lipogenic pathway. Activity differences in numerous lipogenic enzymes in the *Mus* selected lines has been cited (Fell, 1997) as support for the predictions of metabolic control analysis (MCA; also often referred to as metabolic control theory, MCT) (Kacser and Burns, 1979; Kacser and Burns, 1981). This theory proposes that flux control is typically shared by many enzymes of a pathway and that major alterations of flux typically require changes in the activities of many enzymes (termed multisite modulation; see below). However, other cases have been reported in which individual allozymes exert strong flux control (alcohol dehydrogenase and glucose-6-phosphate dehydrogenase; both discussed below).

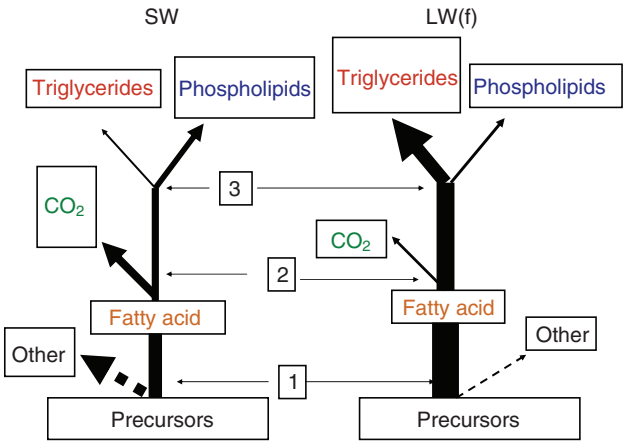

Fig. 2. Diagram comparing the biochemical trade-offs between dispersing (LW(f): long-winged with functional flight muscles) and flightless (SW, short-winged) morphs of *Gryllus firmus* that differ in life history. Width of the lines denotes relative differences between morphs of the flux through pathways of lipid metabolism as determined by radiotracer studies, and the numbers refer to specific trade-offs. Relative to the SW morph, the LW(f) morph diverts a greater amount of lipid precursors into production of fatty acid (trade-off no. 1). Also, less fatty acid is oxidized to CO_2 (trade-off no. 2), and a greater amount is diverted to triglycerides, and less to phospholipids (trade-off no. 3). Data are from Zhao and Zera and Zera and Harshman (Zhao and Zera, 2002; Zera and Harshman, 2009).

Additional studies in *Gryllus* have shown that flux through other pathways in lipid metabolism also differ substantially between the morphs (Zhao and Zera, 2002) (reviewed by Zera and Harshman, 2009) (Fig. 2): in LW(f) individuals, fatty acids are preferentially diverted down the triglyceride rather than the phospholipid arms of glyceride biosynthesis, whereas the opposite occurs in the SW morph. Fatty-acid oxidation is also reduced in the LW(f) compared with the SW genotype. The net result of these differences is the greater production of somatic triglyceride in LW(f) individuals (Fig. 2). Although producing less total fatty acid, the SW morph produces more phospholipid than the LW(f) morph, which may be important in membrane production in developing embryos in the

more fecund SW individuals. Greater oxidation of fatty acid in the SW morph possibly produces energy required to drive the extensive biosynthesis of egg yolk protein in that phenotype. The *Gryllus* study was the first to provide direct support for a long-standing assumption in life-history research: underlying life-history trade-offs are trade-offs in the relative flow of nutrients through pathways of metabolism required to produce end products important for those life histories (Zhao and Zera, 2002).

Many intriguing questions remain unanswered with regard to this research. For example, for *Gryllus* and *Mus* it would be interesting to determine the relative contribution of individual enzymes to total flux differences in lipogenesis in selected lines. Presumably this could be accomplished by sequentially reducing the activities of various enzymes by RNAi. Eanes et al. have recently used single-gene knockouts (P-element excisions) to measure the effect of individual enzymes on glycolytic flux in *D. melanogaster* (Eanes et al., 2006). It would be interesting also to determine the evolutionary trajectory of these extensive metabolic changes. Does this global modification of lipid metabolism evolve in a piecemeal fashion or by a major metabolic change through selection on a single or a few global regulator(s). Laboratory selection indicates that global changes in metabolism can occur relatively rapidly, within a few hundred generations, by a few (<10) individual mutations (Ferea et al., 1999) (discussed below). Importantly, genetic and endocrine studies suggest that the global differences in enzyme activities between LW(f) and SW morphs of *Gryllus* are probably the result of evolutionary changes in endocrine regulation, which coordinates the expression of the lipogenic enzymes (Zera and Zhao, 2003; Zera and Zhao, 2004; Zera and Harshman, 2009). Large-scale temporal changes in flux through metabolic pathways often occur by changes in hormonal regulation that coordinately up- or downregulate numerous enzymes of a pathway, such as the regulation of glycolysis and gluconeogenesis by insulin and glucagon (Granner and Pilkis, 1990). Thus it would not be surprising to see a similar hormonal mechanism responsible for genetic differences in pathway flux within species. Studies of flux changes in metabolic pathways in other species (discussed below) have focused on the influence of independent changes in one or more individual enzymes. The role of evolutionary changes in regulators that coordinately change multiple enzymes of a pathway is a largely unexplored topic in evolutionary studies of metabolism.

Radiotracer and enzyme studies in *Gryllus* have also demonstrated remarkable, large-magnitude morph differences in amino-acid metabolism, which are the mirror image of those seen for lipid metabolism (Zera and Zhao, 2006). Discussion of these data is not possible here because of space constraints (but see Zera and Harshman, 2009). Zera and Harshman also discuss the biochemical and molecular mechanisms responsible for morph differences in activities of lipogenic enzymes (Zera and Harshman, 2011).

Electron transport, hybrid breakdown and speciation

Since the 1930s, the genetic mechanisms responsible for reproductive isolation and speciation have been a central focus of evolutionary biology (Dobzhansky, 1936; Muller, 1942) (reviewed by Coyne and Orr, 2004). The classic model of allopatric speciation proposes that reduced genetic exchange between geographically separated (allopatric) populations leads to genetic divergence and the evolution of population-specific epistatic interactions among alleles at different loci (‘co-adapted gene complexes’). Numerous studies have shown that ‘hybrid breakdown’ commonly occurs in the F_2 generation of interpopulational crosses, which has been interpreted as resulting from the breakdown of these co-adapted complexes as a result of genetic recombination (Coyne and Orr, 2004; Burton, 2006). However, few studies have investigated in detail the functional basis of these negative genetic interactions in natural populations. Recently, Burton and colleagues have conducted a long-term, functional study of F_2 hybrid breakdown, focusing on epistatic interactions between mitochondrial and nuclear genes that encode subunits of enzymes of the mitochondrial electron transport system (ETS).

Burton and co-workers (Rawson and Burton, 2002; Ellison and Burton, 2006; Burton, 2006), and others (e.g. McKenzie et al., 2003; Montooth et al., 2010) have argued that mitochondrial-nuclear interactions provide an excellent model to test hypotheses regarding genetic co-adaptation, coevolution and speciation. Protein complexes of the electron transport system (ETS) are encoded by subunits derived from mitochondrial genes (13 polypeptides) and nuclear genes (70 polypeptides), with four of five complexes containing both nuclear and mitochondrial subunits that function in close association. Because the ETS plays a central role in basic cellular energetics, disruptions in these interactions are expected to have widespread effects on many aspects of growth, reproduction and survival. Because mitochondrial and nuclear genes are inherited separately, individuals derived from different populations can produce F_2 progeny that potentially contain mismatched ETS complex subunits.

The experimental model used by Burton and co-workers is the marine copepod, *Tigriopus californicus*, which occurs in rock outcrops along the western coast of North America. This species exhibits dramatic genetic divergence among populations in both mitochondrial and nuclear genes that produce ETS subunits, as well as genes with other functions [e.g. allozyme genes encoding enzymes of intermediary metabolism (Willet and Burton, 2004; Burton, 2006)]. Substantial genetic differences are stable over decades indicating long-term reduction in genetic exchange among populations. Initial interpopulation crosses demonstrated F_2 hybrid breakdown in *T. californicus*, with F_2 interpopulational hybrids exhibiting slower development and reduced viability and fecundity, compared with F_2 individuals produced from the same population. To directly test the incompatibility hypothesis, Rawson and Burton (Rawson and Burton, 2002) conducted *in vitro* assays involving two interacting components of the ETS complex, cytochrome *c* (CYC), which is composed of a single, nuclear-derived subunit, and cytochome *c* oxidase (COX), which is composed of ten nuclear and three mitochondrial-derived subunits [see figure 1 of Ellison and Burton (Ellison and Burton, 2006)]. Genes encoding each of these mitochondrial components differ substantially (12–32 amino acid substitutions in total) between populations used for the assay [from San Diego and Santa Cruz]. Consistent with the co-adaptation hypothesis, the activity of COX was higher when paired with CYC from its own population than CYC from a different population. There was a difference of three amino acids in the CYC enzymes from the two populations. Harrison and Burton subsequently used site-directed mutagenesis to produce each of the six potential intermediates between the San Diego and Santa Cruz CYC, and identified the amino acids responsible for the reduced activity of the interspecific crosses (Harrison and Burton, 2006). They also directly determined that differences in *in vitro* activity affected organismal fitness by showing that viability was reduced in F_2 hybrids that contained the CYC genotype in the maladaptive mitochondrial background. Finally, they showed that ATP production was reduced in mitochondria from interpopulational

hybrids compared with intrapopulational F_2 individuals, and that rate of ATP production was significantly correlated with survivorship.

These long-term, integrative studies of Burton and colleagues are a wonderful example of how functional studies of intermediary metabolism can provide insights into the functional causes of an important evolutionary process: hybrid breakdown and the evolution of reproductive isolating mechanisms. Burton argues that the results obtained for *T. californicus* may be widespread (Burton, 2006). Genetic differentiation among populations of this species is similar to that found in many organisms, and thus, maladaptive mitochondrial–nuclear interactions leading to reduced mitochondrial function could be an important general cause of F_2 hybrid breakdown. Vertebrate xenomitochondrial cybrids have been produced in which a nucleus from one species is introduced into an enucleated cytoplasm of another species. Even more dramatic reduction in activities of various chimeric ETS complexes (i.e. containing nuclear-encoded subunits from one species and mitochondrial-encoded subunits from other species) have been observed in these vertebrate cybrids, showing that further genetic differentiation leads to even greater disruption of ETS subunit co-adaptation (McKenzie et al., 2003).

Intermediary metabolism and population adaptation in *Drosophila melanogaster*

A number of detailed studies of population genetics have focused on the microevolution of pathways of intermediary metabolism in *Drosophila melanogaster*, an important genetic model in evolutionary research. These studies comprise two major research thrusts. The first and more extensive involves studies of genetic differences in enzymes (allozymes) and pathway features (flux or output) in the context of adaptation of natural populations to different climates. A second approach involves quantitative, genetic analyses of genetic variation in pathway output (e.g. ‘metabolite pools’; glycogen, triglyceride), correlations with underlying enzymatic activities, and effects on fitness components in the laboratory (Clark, 1990) (reviewed by Zera and Harshman, 2011). Because of space constraints, only the first approach will be considered here.

As mentioned previously, initial micoevolutionary studies of intermediary metabolism largely focused on the biochemical properties and fitness consequences of genetic variants of individual enzymes of metabolism (enzyme polymorphism). Subsequent work has increasingly taken on a ‘pathway perspective’, largely influenced by the seminal work of Kacser and Burns (Kacser and Burns, 1979; Kacser and Burns, 1981) (reviewed by Fell, 1997). They showed that substantial variation in the activity of a single enzyme often has only a minimal, if any, effect on *in vivo* flux through the pathway in which the enzyme operates. Thus, it became necessary to directly demonstrate whether kinetic differences between allozymes give rise to corresponding differences in pathway flux. These studies have brought investigations of metabolic pathway function to the forefront of studies of evolutionary biochemistry in *Drosophila*. Because of space considerations I will only briefly summarize two of the most important examples: (1) alcohol dehydrogenase and lipid metabolism, and (2) enzyme polymorphisms at the glucose 6-phosphate (G6P) branchpoint and lipid and glycogen metabolism. This latter example is discussed in detail by Eanes in this issue (Eanes, 2011) and by Zera and Harshman (Zera and Harshman, 2011).

The alcohol dehydrogenase (ADH) polymorphism is probably the most extensively studied enzyme polymorphism in *D. melanogaster*. The two major ADH allozymes, which differ in electrophoretic mobility, differ by only one amino acid substitution (van Delden, 1982; Koehn et al., 1983; Zera et al., 1985; Eanes, 1999). Electrophoretically fast (F) and slow (S) allozymes differ in k_{cat} and differ twofold in the level of protein, although there are no appreciable differences in transcript abundance. Parallel latitudinal clines have been reported in the northern and southern hemispheres of several continents, with the fast (high activity) allele increasing from lower to higher latitudes (colder climates). Extensive studies of nucleotide variation also support the hypothesis that the ADH amino-acid substitution, or a closely linked regulatory site, is a direct target of selection (Eanes, 1999), although the data indicate that the evolutionary history of *Adh* has been complex (Begun et al., 1999). Numerous laboratory studies also indicate that the high-activity ADH-F allozyme has a fitness advantage over the ADH-S allozyme in environments containing ethanol. It should be noted, however, that many genes in addition to *Adh* play a role in adaptation to high alcohol environments (Fry et al., 2004; Fry et al., 2007).

Originally, alcohol tolerance or detoxification was thought to be the ‘agent of selection’ acting on the ADH polymorphism (Stanley and Parsons, 1981). However, subsequent studies have suggested that increased utilization of ethanol for lipid biosynthesis in individuals bearing the fast allozyme might be important (Eanes, 1999). Triglyceride content increases almost 40% from southern to northern US populations of *D. melanogaster*, in parallel with the increase in the frequency of the fast *Adh* allele. Moreover, the polymorphic enzyme, α-glycerophosphate dehydrogenase (α-GPDH), which provides the glycerol backbone for triglyceride biosynthesis, also exhibits a cline in allele frequencies in North America. The allele of the more active form of α-GPDH increases in frequency with latitude, like the more active *Adh* allele, and the activity of both of these enzymes are induced by alcohol in the diet. A parallel cline also exists at the aldehyde dehydrogenase locus (*Aldh*), which encodes the enzyme which acts on acetaldehyde, the product of the ADH reaction (Fry et al., 2007). The high activity *Aldh* allele increases in parallel with the high activity *Adh* allele (Fry et al., 2007).

The ADH polymorphism was one of the first, and remains one of the most extensively studied, enzyme polymorphisms in natural populations with respect to effects of enzyme activity on pathway flux from the perspective of MCA (Kacser and Burns, 1979; Kacser and Burns, 1981; Fell, 1997). These studies illustrate the importance and complexities involved in ascertaining the relationship between enzyme activity and pathway flux.

Ethanol is oxidized to acetaldehyde by ADH, which is then converted to acetyl-CoA by aldehyde dehydrogenase (Fry et al., 2007) which can then be either oxidized to CO_2 for energy or used for the biosynthesis of lipid (Fig. 3). Adult *D. melanogaster* were exposed to [^{14}C]ethanol vapor and the rate of conversion to lipid or CO_2 was monitored (Middleton and Kacser, 1983). Surprisingly, ADH was found to have a very low flux sensitivity coefficient (0.04). A value of 1.0 indicates a 1:1 relationship between enzyme activity and pathway flux (i.e. complete control of pathway flux by that enzyme), whereas a value of 0 means that variation in enzyme activity has no influence of pathway flux, within the range of activities measured. *Adh* genotypes, which differed over more than a threefold range in *in vitro* activity (a difference that spans the range in activity of common allozymes found in natural populations) did not differ significantly with respect to *in vivo* flux from ethanol to CO_2 or lipid during the adult stage. Middleton and Kacser concluded that, despite the large difference in *in vitro*

enzyme activity between *Adh* genotypes, these differences do not result in corresponding differences in pathway flux, during the adult stage (Middleton and Kacser, 1983). Needless to say, this result was puzzling to molecular population geneticists who had long viewed the ADH polymorphism in *D. melanogaster* as the paradigmatic example of an adaptive enzyme polymorphism.

Subsequently, Freriksen et al. found, using ^{13}C nuclear magnetic resonance (NMR), that in *D. melanogaster* larva ADH had a very high flux control coefficient (0.9–1.0), so it was essentially controlling the flux from ethanol to lipid: *Adh* genotypes that differed in enzyme activity differed in *in vivo* conversion of ethanol to triglyceride in a 1:1 ratio (Freriksen et al., 1991) (Fig. 3). These two studies suggest that larval and adult physiologies differ markedly in *D. melanogaster*, and that natural selection may be acting on the ADH polymorphism primarily in larval *Drosophila* (Freriksen et al., 1994). These authors speculated that because ADH activity is higher in adults than in larvae it may exert less control over ethanol metabolism in adults. This study underscores the importance of pathway studies to identify the stage in the life cycle during which natural selection is acting on a genetically variable enzyme.

Subsequently a more comprehensive ^{13}C NMR analysis of ethanol metabolism in *D. melanogaster* larvae was undertaken that identified additional complexities and differences in metabolism between *Adh* genotypes (Freriksen et al., 1994). Ethanol was metabolized through four different pathways (Fig. 3): (1) to lipid; (2) to glutamate and then to glutamine and proline; (3) to malate, pyruvate and finally to lactate and alanine; and (4) to trehalose also *via* malate. As discussed by the authors, conversion to proline and lactate is probably important in the maintenance of redox balance during ethanol oxidation by regenerating NAD^+ by lactate dehydrogenase and proline dehydrogenase. Biosynthesized trehalose could be used by the pentose shunt to generate NADPH for lipid biosynthesis, could be converted into glycogen, or could be used as a cryoprotectant (see below). Finally, conversion of malate to pyruvate could be important in generating NADPH for lipid biosynthesis. Most importantly, Freriksen et al. (Freriksen et al., 1994) observed that, relative to ADH-SS, ADH-FF individuals preferentially metabolized ethanol through malate to trehalose as opposed to lactate and alanine. The authors speculated that this could be adaptive in the ADH-FF genotype, which occurs in higher frequency in northern (colder) latitudes because the increased trehalose, an important cryoprotectant, could increase freeze tolerance. These authors also provided evidence that trehalose is rapidly turned over by the pentose shunt, which might be important for increased NADPH production for increased lipid biosynthesis (however, see studies by Eanes and co-workers below). In addition to identifying these metabolic differences between *Adh* genotypes, the study of Freriksen et al. (Freriksen et al., 1994) is also noteworthy in that it points out some of the unanticipated complexities of metabolic studies. For example, they found that genotypic differences in flux ratios changed over time, and, in some cases, the very existence of genotype differences in flux depended on the time of measurement. Unfortunately, there have been no follow-up studies of the pioneering work of Freriksen et al. (Freriksen et al., 1991; Freriksen et al., 1994).

The glucose-6-phosphate dehydrogenase (G6PD) polymorphism in *D. melanogaster* also has been intensively studied from both functional and evolutionary perspectives (Eanes, 1999). Because this polymorphism has recently been reviewed elsewhere (Zera and Harshman, 2011) and in this volume (Eanes, 2011), only a very brief summary will be given here. The polymorphism mainly

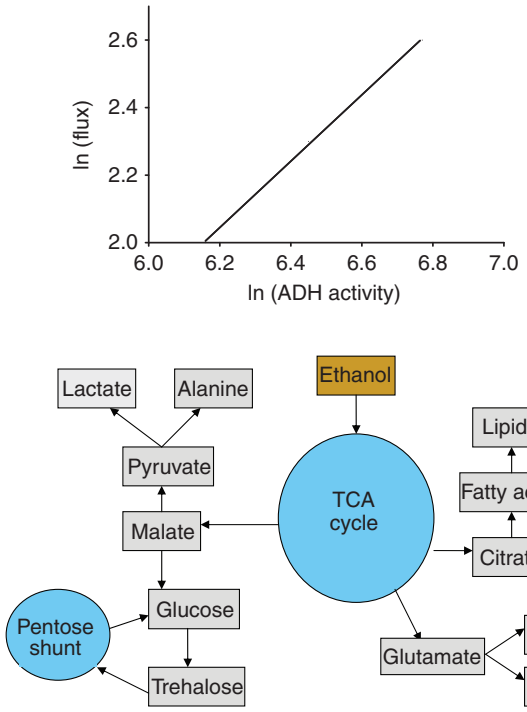

Fig. 3. (Top) Relationship between alcohol dehydrogenase (ADH) activity and flux from ethanol to lipid biosynthesis in larval *Drosophila melanogaster* [modified from fig. 3 of Freriksen et al. (Freriksen et al., 1991)]. The slope of the line (ca. 1.0) is the flux control coefficient of ADH. (Bottom) Various pathways of metabolism of radiolabeled ethanol in larval *D. melanogaster* [modified from fig. 1 of Freriksen et al. (Freriksen et al., 1994)].

consists of two allozymes that vary clinally in North America and Australia, with the low activity allozyme (G6PD-A) increasing in frequency in higher (colder) latitudes. This allozyme exhibits a higher *in vitro* K_m for G6P and lower catalytic efficiency, compared with the 'B' allozyme, and an estimated 20–40% lower activity *in vivo*. Using radioisotopes, Cavener and Clegg, and Labate and Eanes, found that the allele for low-activity G6PD was associated with reduced flux through the pentose shunt (Cavener and Clegg, 1981; Labate and Eanes, 1992). Similar to the situation for the ADH polymorphism, molecular evolution studies provide strong evidence that the G6PD polymorphism is influenced by positive selection (i.e. adaptive clinal variation) (Eanes, 1999). Somewhat paradoxically, natural selection appears to favor decreased pentose shunt flux as a function of increasing latitude (the low activity 'A' allele is more common in northern populations). Because lipid content increases with latitude, and the pentose shunt is thought to provide a significant proportion of NADPH for lipid biosynthesis, one might expect the B allozyme to increase in frequency in northern latitudes.

Verrelli and Eanes argue that an important function of reduced pentose shunt activity, mediated by the A allozyme of G6PD, may actually be to cause diversion of G6P away from lipid biosynthesis and into glycogen biosynthesis (Verrelli and Eanes, 2001a; Verrelli and Eanes, 2001b). According to this hypothesis other NADPH-producing enzymes, such as $NADP^+$ isocitrate dehydrogenase and $NADP^+$ malate dehydrogenase, would play a more important role in providing NADPH for enhanced lipid biosynthesis in diapausing individuals in northern populations (Merritt et al., 2005). Additional support for this interesting idea is the higher frequency,

in northern populations, of a high activity allozyme of the enzyme phosphoglucomutase (PGM), the enzyme involved in the diversion of G6P to glycogen. This high activity allozyme is associated with higher glycogen content in flies. Thus, reduced G6PD and pentose shunt activity, coupled with elevated PGM activity, appear to divert G6P away from lipid and into glycogen (Verrelli and Eanes, 2001b). Availability of the whole genome sequence of *D. melanogaster* allowed the Eanes group to take an even more global genomics approach to the microevolution of glycolysis by investigating the signature of selection on numerous enzymes of the core glycolytic pathway compared with the branchpoint enzymes participating in the metabolism of G6P. Flowers et al. reported that enzyme polymorphisms at the G6PD branchpoint show evidence of being influenced by positive selection to a much greater degree than core enzymes of glycolysis (Flowers et al., 2007). These authors propose that branchpoint enzymes of metabolism are especially important in the evolution of metabolism because they have greater control of pathway flux (for details, see Eanes, 2011). However, simulations by Wright and Rausher (see below) suggest that other differences between these groups of genes, namely whether they occur in the upper or low part of the pathway, may be important in determining the extent to which they control pathway flux and thus are targets of selection (Wright and Rausher, 2010).

An important contribution of the *Drosophila* studies on ADH and G6PD polymorphisms, regarding the microevolution of intermediary metabolism, is that they constitute a detailed dissection of enzymes and pathways of metabolism undertaken in field populations, in the context of geographical variation and climatic adaptation. Most other major analyses of the micoevolution of metabolism have focused on within-population variation (studies by Burton and co-workers, discussed above, are another noteworthy exception). Studies of Eanes and co-workers also constitute a fundamentally important investigation into the relationships among branchpoint location, flux control and enzyme evolution. The *Drosophila* experimental system shows immense promise for identifying how the microevolution of intermediary metabolism contributes to adaptation to different climates.

Chemostat selection and intermediary metabolism

Chemostat selection is a powerful experimental tool to investigate various aspects of biochemical microevolution in the laboratory (Dykhuizen and Dean, 1990; Dykhuizen and Dean, 2009; Rosenzweig and Sherlock, 2009). The power of this approach is that it allows the experimenter to maintain large populations of microorganisms under continual growth conditions for many generations, while maintaining constant population size, under precisely defined experimental conditions. In the context of the present review, chemostat selection has been especially important in, (1) fine-scale studies of natural selection acting on a number of enzymes of specific pathways, and (2) long-term evolutionary studies (hundreds of generations) to identify pathway adaptations to specific environments.

Dykhuizen, Dean and colleagues undertook a series of landmark chemostat studies to investigate the action of natural selection on enzymes of lactose catabolism in *Escherichia coli* (Dykuizen and Dean, 1990; Dykuizen and Dean, 2004; Dykuizen and Dean, 2009). These studies were among the first to apply MCA (Kacser and Burns, 1979; Kacser and Burns, 1981) to population genetics, and to show, in a rigorous, quantitative way, how the relative fitness values of enzyme genotypes were dependent on the extent to which they differentially influenced pathway flux.

Lactose catabolism in *E. coli* is a relatively simple pathway involving a porin pore, which allows passive diffusion of lactose through the outer cell wall into the periplasm, a permease which transports lactose into the cytoplasm, and β-galactosidase, which cleaves lactose into glucose and galactose. In their experiments, fitness (growth of a strain relative to other competing strains) was directly proportional to flux through the pathway of catabolism of lactose, the limiting nutrient (Fig. 4). Using a variety of genetic variants of these pathway components they showed that natural variation in the activity of β-galactosidase had almost no effect on pathway flux (Fig. 4). By contrast, variation in permease activity, and, to a much greater degree, porin size, did influence pathway flux. These studies provided a mechanistic explanation of why naturally occurring variants of β-galactosidase are neutral with respect to fitness, and thus are not acted on by natural selection. Furthermore, these studies predicted that evolutionary changes in lactose catabolism would occur primarily by natural selection targeting the permease and porin genes, predictions that were subsequently confirmed (Dykhuizen and Dean, 2009). Another important message of the Dykuizen and Dean studies is that fitness differences between genotypes of enzyme ‘X’ in the pathway are not fixed, but are dependent upon other characteristics of the pathway, specifically the extent to which flux is controlled by enzyme ‘X’, relative to other enzymes in the pathway. They argue that, if natural selection were to cause a dramatic increase in the activity of porin pores (increase size), flux control would shift more to the permease and β-galactosidase enzymes. β-galactosidase allozymes, which previously were neutral because they did not differentially affect flux, might now affect flux and thus be exposed to natural selection [however, see Wright and Rausher (Wright and Rausher, 2010) and ‘Concluding remarks’ for a critique of this idea]. The importance of the work of Dykuizen and Dean on the lactose catabolic pathway of *E. coli* is that they provide a quantitative, mechanistic understanding of key concepts in population genetics, such as fitness, selection, constraint, epistasis and gene-X–environment interaction in terms of enzyme function in biochemical pathways.

As stated above, chemostats also have been used extensively to investigate long-term evolutionary changes over hundreds to thousands of generations (reviewed by Rosenzweig and Sherlock, 2009). An excellent example of this approach is the study of Ferea et al. (Ferea et al., 1999) that was among the first to use microarray analyses to investigate adaptive, global changes in pathways of intermediary metabolism under laboratory selection. Building on prior physiological studies of laboratory selection (Paquin and Adams, 1983), Ferea et al. (Ferea et al., 1999) subjected a single *Saccharomyces cerevisiae* diploid clone to replicate selection by aerobic growth on glucose-limited medium for approximately 250 generations. They then profiled the yeast transcriptome of the two replicates of their study, as well as the unevolved parental strain, and another strain that had been subjected to the same environmental conditions but for a longer period of time (500 generations) from a study by Paquin and Adams (Paquin and Adams, 1983). They found remarkable, consistent systemic responses to selection in each of the three selected lines relative to the unselected parental line. The genes encoding enzymes of glycolysis were downregulated, especially those of the terminal steps leading to alcohol production. By contrast, the genes encoding the enzymes of the tricarboxylic acid cycle, oxidative phosphorylation, electron transport and glucose transport were all elevated. The general picture is the systemic evolutionary modification of major blocks of carbohydrate metabolism, in which

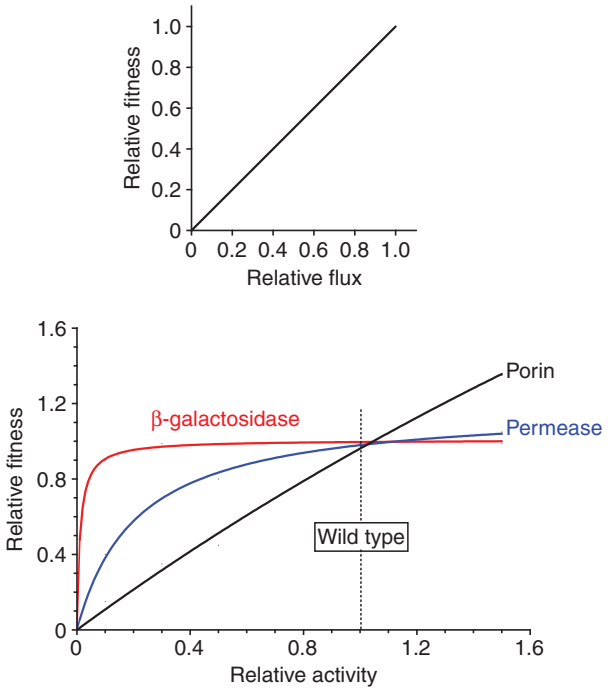

Fig. 4. (Top) Relationship between flux through the pathway of lactose catabolism and fitness (growth rate) in various strains of *Escherichia coli* in a lactose-limited chemostat. Note the 1:1 relationship between flux and growth rate. (Bottom) Relationship between enzyme activity and flux through the pathway of lactose catabolism (or growth rate) for strains of *E. coli* differing only at the locus encoding the porin protein (black line), the permease transporter (blue line), or the β-galactosidase enzyme (red line). Note that because naturally occurring alleles of β-galactosidase produce allozymes whose activities occur on the plateau of the flux–activity graph (i.e. near the 'Wild type' value), variation in activity among these alleles has only a small effect on pathway flux. By contrast, variation in permease, and especially porin, alleles has a much larger effect on pathway flux [modified from Dykuizen and Dean (Dykuizen and Dean, 2009)].

fermentation of glucose is inhibited in favor of full aerobic metabolism of glucose. Because the ATP yield is much greater when glucose is metabolized aerobically, the evolved strains were essentially adapted for more efficient glucose utilization. Given the number of generations of selection, only a relatively few mutations (ca. 10) were expected to have caused the observed changes in hundreds of gene transcripts. Thus, the authors speculated that the systemic changes in carbohydrate metabolism were probably caused by mutation of only a few key genes possibly encoding regulators of metabolism. Subsequent characterization (Dunham et al., 2002) of some of these evolved strains, and evolved strains from the original study of Paquin and Adams (Paquin and Adams, 1983) identified amplification of the chromosomal arm containing the hexose transporter gene in several clones, and a breakpoint in a chromosome near the gene for mitochondrial citrate synthase, an enzyme that significantly influences flux through the tricarboxylic acid cycle. The authors speculated that the chromosomal alteration may have changed the regulation of citrate synthase leading to some of the observed systemic changes in metabolism. Of course, alterations in other regulators of carbohydrate metabolism could also be involved. Recent population genomic and transcriptome studies in yeast suggest that changes in gene expression are a common mode of adaptive evolution in budding yeast, sometimes accounting for changes in the expression of whole pathways (Fraser et al., 2010). These yeast studies collectively illustrate the significant impact various omics approaches is having on investigations of pathway microevolution. An important caveat of transcriptome studies is that variation in gene expression does not necessarily result in changes in protein levels (Laurie and Stam, 1988; Ideker et al., 2001; Feder and Walser, 2005) (see below). Additional studies are required to address this point (see Concluding remarks).

Microevolution of the anthocyanin pathway and flower color

Integrative ecological, physiological, biochemical and molecular-genetic studies focusing on the evolution of anthocyanin floral pigments have recently developed into a powerful experimental model for investigating a number of central issues in evolution and adaptation (Clegg and Durbin, 2003; Ehrenreich and Purugganan, 2006; Rausher, 2008; Streisfeld and Rausher, 2009; Wright and Rausher, 2010). Ecological aspects of flower color evolution have been extensively investigated (e.g. color preference of pollinators; pleiotropic effects of color alleles), the pathway that produces various anthocyanin pigments is conserved thus allowing comparative studies to be undertaken, and the pathway also has been extensively investigated from physiological, biochemical and molecular perspectives, thus providing the background for mechanistic studies of evolution (Shirley, 1996; Clegg and Durbin, 2003) [Rausher (Rausher, 2008) and references therein]. Here we will limit the focus to recent work undertaken by Rausher and co-workers on evolutionary changes in the anthocyanin pathway in *Ipomoea* (morning glory; Convolvulaceae) and *Iochroma* (Solanaceae).

A simplified version of the anthocyanin pathway is given in Fig. 5. Basically, the enzyme chalcone synthase (CHS) controls the first committed step in flavonoid biosynthesis. The key intermediate, dihydrokaempherol (DHK), can be converted to three products, each of which is subsequently metabolized down separate pathways, giving rise to three different anthocyanin pigments: delphinidin (mainly purple or violet), cyanidin (mainly blue or magenta) or pelargonidin (mainly red or orange). Importantly, delphinidin is not found in the *Ipomoea* species studied by Rausher and thus alterations in only the cyanidin and pelargonidin arms of the pathway need to be considered in species of this genus, whereas modifications in each of the three arms need to be considered in *Iochroma*. Three enzymes [dihydroflavonol-4-reductase (DFR), anthocyanin synthase (ANS) and UDP-flavonoid-3-glucosyl-transferase (UF3GT)] each catalyze reactions in each of the three arms of the anthocyanin pathway. Thus, evolutionary changes in relative flux down the various arms of the flavonoid pathway can occur because of changes in the relative specificities of these enzymes for flavonoids unique to each arm, or to changes in the activity or specificity of enzymes at branch points [e.g. flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H)]. It is important to bear in mind that the anthocyanin pathway is complex and produces products other than plant pigments, such as condensed tannins and nonpigmented flavonoids, and that flavonoids play roles other than as pigments, which are important in pollinator attraction (Shirley, 1996; Quattrocchio et al., 2006; Rausher, 2008).

Biochemical and molecular mechanisms of parallel evolution

Similar phenotypes often evolve independently, and the mechanisms involved in parallel evolution have been of considerable interest to evolutionary biologists. Are there preferred pathways of evolution? To what extent does parallel evolution

occur by modification of similar points in pathways or networks? Rausher and co-workers investigated this issue in three species of *Ipomoea* (Streisfeld and Rausher, 2009; Des Marais and Rausher, 2010) and in two species of *Iochroma* (S. D. Smith and M. D. Rausher, personal communication). In the three species of *Ipomoea*, red flower pigment evolved independently from the ancestral blue–magenta anthocyanin pigment by transcriptional, tissue-specific downregulation of the same enzyme, F3′H (Fig. 5), and consequent diversion of flux down the pelargonidin branch of the pathway and away from cyanin production (recall that the delphinidin branch does not operate in these *Ipomoea* species). Changes in the activities and/or specificity of downstream enzymes (e.g. DFR) do not appear to have played a role. Smith and Rausher also found that red color in *Iochroma gesneriodes* evolved from the ancestral blue color by tissue-specific downregulation of F3′H (S. D. Smith and M. D. Rausher, personal communication). In addition they found a deletional inactivation of F3′5′H, as well as a substantial change in substrate specificity in DFR, which they propose improves specificity of the enzyme to pigment precursors of the pelargonidin pathway. Importantly, the mode of action of genetic variants involved in evolutionary changes in flower color (tissue-specific changes in gene regulation) differ from that of red-flower mutants segregating within populations (loss of function in the sequence encoding F3′H; data cited in Streisfeld and Rausher (Streisfeld and Rausher, 2009). Streisfeld and Rausher (Streisfeld and Rausher, 2009), Des Marais and Rausher (Des Marais and Rausher, 2010) and S. D. Smith and M. D. Rausher (personal communication) suggest that regulatory mutations have been preferentially selected in flower-color evolution because they have less negative pleiotropic effects, in that they change anthocyanin expression only in flowers (see also Streisfeld and Rausher, 2010). These studies are a unique contribution to understanding an important, but understudied, topic in pathway micoevolution: relative contribution of evolutionary changes in regulatory *versus* structural (enzyme-encoding) genes underlying evolutionary modification of pathway flux. The relative contribution to adaptive evolution of mutations in *cis*-acting regulatory regions *versus* mutations that alter the amino acid sequence in structural genes remains a contentious issue [see Hoekstra and Coyne (Hoekstra and Coyne, 2007) and references therein].

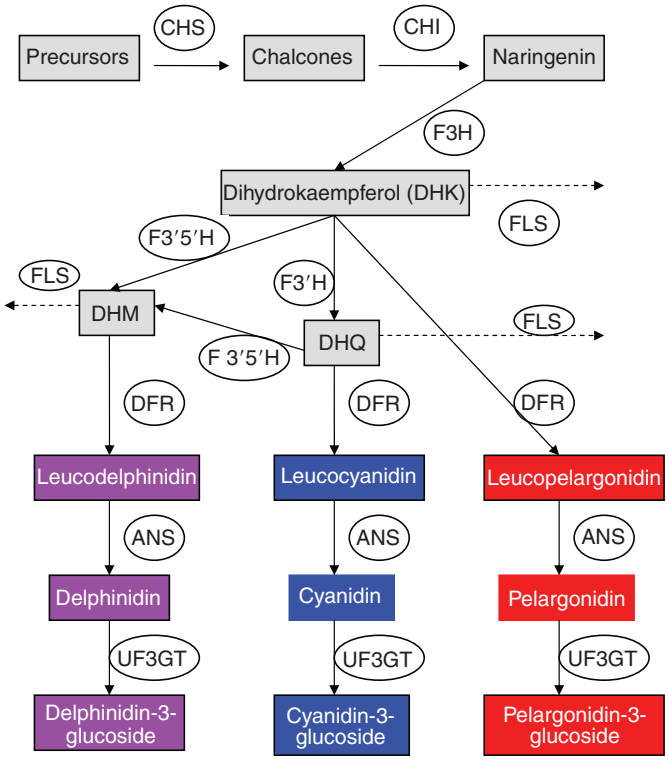

Fig. 5. Simplified version of the anthocyanin pigment pathway in *Ipomoea* and *Iochroma*, which produces three pigments, delphinidin (mainly purple and violet), cyanidin (mainly blue), and pelargonidin (mainly red). Metabolites and products are in shaded boxes and enzymes are circled. Cyanidin and pelargonidin but not delphinidin pigments occur in *Ipomoea* (i.e. the delphinidin portion of the pathway is non-functional in this genus), whereas all three pigments occur in *Iochroma*. The enzymes DFR, ANS and UF3GT act on pigment precursors in each of the three arms of the anthocyanin pathway. Dashed lines indicate pathways to other flavonoids. Modified from figure 1 of Streisfeld and Rausher (Streisfeld and Rausher, 2009). ANS, anthocyanin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercitin; F3H, flavone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; UF3GT, UDP-flavonoid-3-glucosyl-transferase.

Mechanisms by which adaptation constrains future evolution

Studies discussed above are also relevant to another important evolutionary issue: the ‘irreversibility’ of evolution. Many phylogenetic studies suggest that adaptive change can constrain the future direction of evolution (Zufall and Rausher, 2004). However, the mechanistic basis of this phenomenon is not well understood, although plausible hypotheses have been put forward. For example, once one gene is altered to eliminate the expression of a multigenic trait, other genes that influence the expression of the phenotype can accumulate mutations because these mutations are no longer ‘visible’ to natural selection. The accumulation of many mutations makes re-evolving the lost trait very difficult because this would require simultaneous alterations in many genes. The evolution of a red flower from the ancestral blue flower in species of *Iochroma* illustrates this point. The evolution of a red flower in *I. gesnerioides* is associated with blockages at three points in the anthocyanin pathway: one involves deletion of the gene coding for the anthocyanin pathway enzyme F3′5′H (only one functional copy of this gene occurs in *I. gesnerioides*), another involves a change in the expression of F3′H, and another involves a change in specificity of DFR (see Fig. 5) (S. D. Smith and M. D. Rausher, personal communication). This study not only illustrates the importance of gene loss in adaptive evolution, it also shows that re-evolution of a blue flower in *I. gesnerioides*, especially by production of delphinidin pigment, would be highly unlikely.

Pathway position and rate of evolution

Rate of protein evolution varies over several orders of magnitude, and an important evolutionary issue is the cause of this enormous variability. During the past decade, influence of pathway location (upstream *versus* downstream; branchpoint *versus* core) on the rate of evolution has become an important focus of studies of protein evolution, with enzymes in the anthocyanin pathway playing a prominent role in these investigations (Rausher et al., 1999; Rausher et al., 2008; Wright and Rausher, 2010). Metabolic pathways often show downstream branching (e.g. Fig. 5) and the enzymes at the upper part of the pathway are expected to exhibit more pleiotropic interactions (more network connectivity) than enzymes below the branchpoints because they influence more downstream products. A number of workers have proposed that

evolution of these more pleiotropic enzymes should be more constrained, and thus should evolve more slowly relative to enzymes in the more downstream portions of a pathway. Rausher et al. provided support for this hypothesis: three upstream enzymes involved in anthocyanin synthesis evolve more slowly than three downstream enzymes (Rausher et al., 1999). Subsequent studies reported that the increased rate of evolution of the downstream enzymes was due to relaxation of a selective constraint rather than stronger positive selection, which is also consistent with the aforementioned hypothesis (Lu and Rausher, 2003; Rausher et al., 2008). A more extensive statistical analysis of genes of the terpenoid pathway of plants, in five fully sequenced angiosperms, also showed that enzymes in the upper part of pathways evolve more slowly than those in the lower portion (Ramsay et al., 2009). Finally, recent models of evolutionary change in flux through a linear pathway (Wright and Rausher, 2010) also predict that control will evolve to be highly unequal among enzymes of a linear pathway, with upstream enzymes evolving greater control. The work of Rausher and co-workers illustrates how studies of metabolic pathways can be used to investigate long-standing issues in evolution. These studies also provide important insights into the influence of pathway properties on rate of protein evolution.

Concluding remarks

Recent evolutionary studies of intermediary metabolism, described above, have made important contributions to our understanding of evolutionary mechanisms in general, and mechanisms of biochemical evolution in particular. In some cases (e.g. ADH and G6PD polymorphisms in *D. melanogaster*), these studies have deepened our understanding of longstanding issues in biochemical evolution, such as the evolutionary dynamics of enzyme polymorphism. Studies of metabolism have provided key information on the chain of causality by which molecular and enzymatic variation gives rise to variation in pathway flux, and subsequently to variation in whole-organism traits (e.g. energy reserves). In other cases, evolutionary studies of metabolic pathways are providing mechanistic insights into evolutionary topics that have not previously been extensively investigated from a biochemical perspective, such as hybrid breakdown and speciation, parallel evolution, and life-history evolution. Finally, investigations of pathways of intermediary metabolism are providing new insights into the role of pathway properties in the evolution of component parts, such as the influence of pathway position on rate of enzyme evolution.

Development of more advanced models of pathway evolution will certainly be one of the most important areas of future research on the microevolution of metabolic pathways. Although various theoretical treatments of metabolism, most notably MCA (Kacser and Burns, 1979; Kacser and Burns, 1981; Fell, 1997), have contributed enormously to our conception of pathway microevolution, many important topics in metabolism have yet to be investigated in detail from this or other theoretical perspectives. For example, MCA has largely focused on linear pathways of metabolism, and the extent to which results of these analyses also apply to branched pathways, which are common in metabolism, is uncertain (Wright and Rausher, 2010). A number of workers (Eanes, 1999; Eanes, 2011; Wright and Rausher, 2010) have argued that branchpoint enzymes should be a prime focus of future theoretical and experimental studies of pathway evolution.

The degree to which important conclusions derived from MCA are dependent upon specifics of the model is currently a matter of debate. For example, Savageau and Sorriba, and Bagheri and Wagner argue that the key conclusion of Kacser and Burns, that dominance is an inherent property of enzymes linked in a metabolic pathway, is, in fact, a consequence of simplifying assumptions of the MCA model (Savageau and Sorribas, 1989; Bagheri and Wagner, 2004; Kacser and Burns, 1981). However, simulations by Wright and Rausher, which investigated MCA as well as other models of metabolism, suggest that dominance is an inherent property of many of the enzymes of a pathway, independent of assumptions of a specific model (Wright and Rausher, 2010). More recent simulations of branched pathways by M. D. Rausher (personal communication) have lead to a similar conclusion. In a related example, Dykuizen and co-workers (e.g. Dykuizen and Dean, 2009), using an MCA perspective, argued that evolution will cause the control of pathway flux to shift among different enzymes over time, simply as a consequence of inherent systemic interactions of pathway enzymes (discussed above). However, Wright and Rausher argue that this conclusion is dependent upon the assumption of those authors that pathway flux evolves under directional selection; no such shift occurs in simulations of Wright and Rausher (Wright and Rausher, 2010) under stabilizing selection.

A variety of regulatory issues also warrant increased attention in future studies. The first is the extent to which evolutionary change in whole pathway flux occurs through changes in systemic pathway regulators. According to MCA, the optimal strategy for changing flux through a pathway without incurring deleterious changes in levels of intermediates, is by co-ordinate changes in the activities of many enzymes of the pathway (Fell and Thomas, 1995; Fell, 1997). Considerable evidence indicates that this mechanism, termed multisite modulation, is a common mode of flux regulation by environmentally induced systemic regulators (e.g. hormonal regulation of glycolysis and gluconeogenesis). As discussed above, this mechanism could also be a common mode of adaptive evolutionary change in pathway flux, but endocrine–genetic regulation of pathway flux has thus far only been investigated in any detail in a few cases, such as lipid metabolism and life-history morphs of *Gryllus* (Zhao and Zera, 2002; Zera, 2005) (Fig. 2). This topic deserves more extensive study in the future. A second related issue, the extent to which changes in regulatory *versus* structural (protein-encoding) genes contribute to evolutionary changes in pathway flux, also has not been extensively studied. The notable exceptions are the landmark investigations of tissue-specific changes in the regulation of branchpoint enzymes controlling flower pigment biosynthesis by Rausher and colleagues (see section above on ‘Biochemical and molecular mechanisms of parallel evolution’). A key issue for the future should be the conditions that select for modification in tissue-specific regulation, systemic regulation or enzyme function.

Finally, the increasing flood of molecular and biochemical data from various omics approaches (transcriptome profiling, proteomics) (van Straalen and Roelofs, 2006; Pagel and Pomiankowski, 2008) will also undoubtedly increase focus on the microevolution of metabolism as a key topic in evolutionary research. This wealth of omics data on pathway components is providing the material for more intensive and extensive studies of various aspects of pathway evolution, in both model organisms [e.g. yeast (Ferea et al., 1999) *Drosophila* (Larracuente et al., 2007; Gershman et al., 2007; Greenburg et al., 2008; Matzkin and Markow, 2009)], and non-model species (Powell, 2003; Gracey and Cossins, 2003; Gracey, 2003; St-Cyr et al., 2008). However, it is well known that variation in transcript abundance, or enzyme concentration and/or activity, does not necessarily give rise to

variation at a higher physiological level (e.g. pathway flux) (Laurie and Stam, 1988; Ideker et al., 2001; Feder and Walser, 2005; Feder, 2007; Dykuizen and Dean, 2009) (Fig. 4). Thus, studies of pathway function also will play an increasing indispensable role in evaluating the functional and fitness consequences of transcriptomic and proteomic variation.

Acknowledgements

This paper is based on a talk given at the 2010 *Journal of Experimental Biology* Symposium on 'The Biology of Energy Expenditure', held in Mürren, Switzerland. I thank the organizers of the symposium for inviting me to participate. I also thank W. F. Eanes, M. D. Rausher, J. Storz and two anonymous reviewers for thoughtful comments on a previous version of the manuscript. Finally, I gratefully acknowledge the National Science Foundation for continuous support of my research during the past 20 years, most recently grants IOS-0516973 and IBN-0212486.

References

Asante, E. A., Hill, W. G. and Bulfield, G. (1989). Analysis of lines of mice selected for fat content. 1. Correlated responses in the activities of NADPH-generating enzymes. *Genet. Res.* **54**, 155-160.

Asante, E. A., Hill, W. G. and Bulfield, G. (1991). Analysis of lines of mice selected for fat content. 3. Flux through the *de novo* lipid synthesis pathway. *Genet. Res.* **58**, 123-127.

Bagheri, H. C. and Wagner, G. P. (2004). Evolution of dominance in metabolic pathways. *Genetics* **168**, 1713-1735.

Baldwin, E. (1970). *An Introduction to Comparative Biochemistry*. Cambridge: Cambridge University Press.

Begun, D., Betancourt, A. J., Langley, C. H. and Stephan, W. (1999). Is the fast/slow allozyme variation at the *Adh* locus of *Drosophila melanogaster* an ancient balanced polymoprhism? *Mol. Biol. Evol.* **16**, 1816-1819.

Burton, R. S. (2006). The sorry state of F_2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am. Nat.* **168**, S14-S24.

Cavener, D. R. and Clegg, M. T. (1981). Evidence for biochemical and physiological differences between enzyme genotypes in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **78**, 4444-4447.

Clark, A. G. (1990). Genetic components of variation in energy storage in *Drosophila melanogaster*. *Evolution* **44**, 637-650.

Clegg, M. T. and Durbin, M. L. (2003). Tracing floral adaptions from ecology to molecules. *Nat. Rev. Genet.* **4**, 206-215.

Coyne, J. A. and Orr, H. A. (2004). *Speciation*. Sunderland, MA: Sinauer.

Des Marais, D. L. and Rausher, M. D. (2010). Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in Ipomoea. *Evolution* **64**, 2044-2054.

Dobzhansky, T. (1936). Studies on hybrid sterility. II. Localization of sterility factors in *Drosophila pseudoobscura*. *Genetics* **21**, 113-135.

Dunhan, M. J., Badrane, H., Ferea, T., Adams, J., Brown, P. O., Rosenzweig, F. and Botstein, D. (2002). Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99**, 16144-16149.

Dykhuizen, D. E. and Dean, A. M. (1990). Enzyme activity and fitness: evolution in solution. *Trends Ecol. Evol.* **5**, 257-262.

Dykhuizen, D. E. and Dean, A. M. (2004). Evolution of specialists in an experimental microcosm. *Genetics* **167**, 2015-2026.

Dykhuizen, D. E. and Dean, A. M. (2009). Experimental evolution from the bottom up. In *Experimental Evolution. Concepts, Methods, and Applications of Selection Experiments* (ed. T. Garland, Jr and M. R. Rose), pp. 67-87. Berkeley: University of California Press.

Eanes, W. F. (1999). Analysis of selection on enzyme polymorphisms. *Annu. Rev. Ecol. Syst.* **30**, 301-326.

Eanes, W. F. (2011). Molecular population genetics and selection in the glycolytic pathway. *J. Exp. Biol.* **214**, 165-171.

Eanes, W. F., Merritt, T. J. S., Flowers, J. M., Kumagai, S., Sezgin, E. and Zhu, C.-T. (2006). Flux control and excess capacity in the enzymes of glycolysis and their relationship to flight metabolism in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **103**, 19413-19418.

Ehrenreich, I. M. and Purugganan, M. D. (2006). The molecular genetic basis of plant adaptation. *Am. J. Bot.* **93**, 953-962.

Ellison, C. K. and Burton, R. S. (2006). Distruption of mitochondrial function in interpopulational hybrids of *Tigriopus californicus*. *Evolution* **60**, 1382-1391.

Feder, M. E. (2007). Evolvability of physiological and biochemical traits: evolutionary mechanisms including and beyond single-nucleotide mutation. *J. Exp. Biol.* **210**, 1653-1660.

Feder, M. E. and Walser, J.-C. (2005). The biological limitations of transcriptomics in elucidating stress and stress responses. *J. Evol. Biol.* **18**, 901-910.

Feder, M. E. and Watt, W. B. (1992). Functional biology of adaptation. In *Genes in Ecology* (ed. R. J. Berry, T. J. Crawford and G. M. Hewitt), pp. 365-392. Oxford: Blackwell Scientific Publications.

Fell, D. (1997). *Understanding the Control of Metabolism*. London: Portland Press.

Fell, D. and Thomas, T. (1995). Physiological control of metabolic flux: the requirement for multisite modulation. *Biochem. J.* **311**, 35-39.

Ferea, T. L., Botstein, D., Brown, P. O. and Rosenzweig, R. F. (1999). Systematic changes in gene expression patterns following adaptive evolution in yeast. *Proc. Natl. Acad. Sci. USA* **96**, 9721-9726.

Flowers, J., Sezgin, E., Kumagai, S., Duvernell, D., Matzkin, L., Schmidt, P. and Eanes, W. F. (2007). Adaptive evolution of metabolic pathways in *Drosophila*. *Mol. Biol. Evol.* **24**, 1347-1354.

Fraser, H. B., Moses, A. M. and Schadt, E. E. (2010). Evidence for widespread adaptive evolution of gene expression in budding yeast. *Proc. Natl. Acad. Sci. USA* **107**, 2977-2982.

Freriksen, A., Seykens, D., Scharloo, W. and Heinstra, P. W. H. (1991). Alcohol dehydrogenase controls the flux from ethanol into lipids in *Drosophila* larvae. A ^{13}C NMR study. *J. Biol. Chem.* **266**, 21399-21403.

Freriksen, A., de Ruiter, B. L. A., Scharloo, W. and Heinstra, P. W. H. (1994). *Drosophila* alcohol dehydrogenase polymorphism and carbon-13 fluxes: Opportunities for epistasis and natural selection. *Genetics* **137**, 1071-1078.

Fry, J. D., Bahnck, C. M., Mikucki, M., Phadnis, N. and Slattery, W. C. (2004). Dietary ethanol mediates selection on aldehyde dehydrogenase activity in *Drosophila melanogaster*. *Integr. Comp. Biol.* **44**, 275-283.

Fry, J. D., Donlon, K. and Saweikis, M. (2007). A worldwide polymorphism in aldehyde dehydrogenase in *Drosophila melanogaster*: Evidence for selection mediated by dietary ethanol. *Evolution* **62**, 66-75.

Gershman, B., Puig, O., Hang, L., Peitzsch, R. M., Tatar, M. and Garofalo, R. S. (2007). High resolution dynamics of the transcriptional response to nutrition in *Drosophila*: a key role for dFOXO. *Physiol. Genomics* **29**, 24-34.

Gracey, A. (2003). Interpreting physiological responses to environmental change through gene expression profiling. *J. Exp. Biol.* **209**, 1584-1592.

Gracey, A. and Cossins, A. (2003). Application of microarray technology in environmental and comparative physiology. *Annu. Rev. Physiol.* **65**, 231-259.

Granner, D. and Pilkis, S. (1990). The genes of hepatic glucose metabolism. *J. Biol. Chem.* **265**, 10173-10176.

Greenberg, A. J., Stockwell, S. R. and Clark, A. G. (2008). Evolutionary constraint and adaptation in the metabolic network of *Drosophila*. *Mol. Biol. Evol.* **25**, 2537-2546.

Hall, J. G. and Koehn, R. K. (1983). The evolution of catalytic efficiency and adaptive inference from steady-state kinetic data. *Evol. Biol.* **16**, 53-96.

Harrison, J. S. and Burton, R. S. (2006). Tracing coadapted gene complexes to single amino acid substitutions. *Mol. Biol. Evol.* **23**, 559-564.

Harshman, L. G. and Schmidt, J. L. (1998). Evolution of starvation resistance in *Drosophila melanogaster*: aspects of metabolism and counter-impact selection. *Evolution* **52**, 1679-1685.

Harshman, L. G. and Zera, A. J. (2007). The cost of reproduction: the devil in the details. *Trends Ecol. Evol.* **22**, 80-86.

Hastings, I. M. and Hill, W. G. (1990). Analysis of lines of mice selected for fat content. 2. Correlated responses in the activities of enzymes involved in lipogenesis. *Genet. Res.* **55**, 55-61.

Hochachka, P. W. (1973). Comparative intermediary metabolism. In *Comparative Animal Physiology* (ed. C. L. Prosser), p. 212. Philadelphia: W. B. Saunders.

Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation. Mechanism and Process in Physiological Evolution*. Oxford: Oxford University Press.

Hoekstra, H. E. and Coyne, J. A. (2007). The locus of evolution: evo devo and the genetics of adaptation. *Evolution* **61**, 995-1016.

Ideker, T., Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., Bumgarner, R., Goodlett, D. R., Aebersold, R. and Hood, L. (2001). Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* **272**, 929-934.

Kacser, H. and Burns, J. A. (1979). Molecular democracy: who shares the controls. *Trans. Biochem. Soc.* **7**, 1149-1160.

Kacser, H. and Burns, J. A. (1981). The molecular basis of dominance. *Genetics* **97**, 639-666.

Koehn, R. K., Zera, A. J. and Hall, J. G. (1983). Enzyme polymorphism and natural selection. In *Evolution of Genes and Proteins* (ed. M. Nei and R. K. Koehn), pp. 115-136. Sunderland, MA: Sinauer.

Labate, J. and Eanes, W. F. (1992). Direct measurement of in vivo flux differences between electrophoretic variants of G6PD in *Drosophila melanogaster*. *Genetics* **132**, 783-787.

Larracuente, A. M., Sackton, T. B., Greenberg, A. J., Wong, A., Singh, N. D., Sturgill, D., Zhang, Y., Oliver, B. and Clark, A. G. (2007). Evolution of protein-coding genes in *Drosophila*. *Trends Genet.* **24**, 114-123.

Laurie, C. C. and Stam, L. (1988). Quantitative analysis of RNA produced by Slow and Fast alleles of *Adh* in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **85**, 5161-5165.

Lewontin, R. (1974). *The Genetic Basis of Evolutionary Change*. New York: Columbia University Press.

Lu, Y. and Rausher, M. D. (2003). Evolutionary rate variation in anthocyanin pathway genes. *Mol. Biol. Evol.* **20**, 1844-1853.

Matzkin, L. and Markow, T. (2009). Transcriptional regulation of metabolism associated with the increased desiccation resistance of the cactophilic *Drosophila mojavensis*. *Genetics* **182**, 1279-1288.

McKenzie, M., Chiotis, M., Pinkert, C. A. and Trounce, I. A. (2003). Functional respiratory chain analyses in murid xenomitochondrial cybrids expose coevolutionary constraints of cytochrom b and nuclear subunits of complex III. *Mol. Biol. Evol.* **20**, 1117-1124.

Merritt, T. J. S., Duvernell, D. and Eanes, W. F. (2005). Natural and synthetic alleles provide complementary insights into the nature of selection acting on the *Men* polymorphism of *Drosophila melanogaster*. *Genetics* **171**, 1707-1718.

Middleton, R. J. and Kacser, H. (1983). Enzyme variation, metabolic flux and fitness: Alcohol dehydrogenase in *Drosophila melanogaster*. *Genetics* **105**, 633-650.

Mitton, J. B. (1997). *Selection in Natural Populations*. Oxford: Oxford University Press.

Montooth, K. L., Meiklejohn, C. D., Abt, D. N. and Rand, D. M. (2010). Mitochondrial-nuclear epistasis affects fitness within but does not contribute to fixed incompatibilities between species of *Drosophila*. *Evolution* doi:10.1111/j.1558-5646.2010.01077.x.

Muller, H. J. (1942). Isolating mechanisms, evolution, and temperature. *Biol. Symp.* **6**, 71-125.
Pagel, M. and Pomiankowski, A. (2008). *Evolutionary Genomics and Proteomics*. Sunderland, MA: Sinauer Associates.
Paquin, C. and Adams, J. (1983). Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. *Nature* **30**, 495-500.
Powell, F. L. (2003). Functional genomics and the comparative physiology of hypoxia. *Annu. Rev. Physiol.* **65**, 203-230.
Quattrocchio, F., Baudry, A., Lepiniec, L. and Grotewold, E. (2006). The regulation of flavonoid biosynthesis. In *The Science of Flavonoids* (ed. E. Grotewold), pp. 97-122. Heidelberg: Springer.
Ramsay, H., Reisberg, L. H. and Ritland, K. (2009). The correlation of evolutionary rate with pathway position in plant terpenoid biosynthesis. *Mol. Biol. Evol.* **26**, 1045-1053.
Rausher, M. D. (2008). Evolutionary transitions in floral color. *Int. J. Plant Sci.* **169**, 7-21.
Rausher, M. D., Miller, R. E. and Tiffin, P. (1999). Patterns of evolutionary rate variation among genes of the anthocyanin biosynthetic pathway. *Mol. Biol. Evol.* **16**, 266-274.
Rausher, M. D., Yingqing, L. and Meyer, K. (2008). Variation in constraint versus positive selective as an explanation for evolutionary rate variation among anthocyanin genes. *J. Mol. Evol.* **67**, 137-144.
Rawson, P. D. and Burton, R. S. (2002). Functional coadaptation between cytochrome *c* and cytochrom *c* oxidase within allopatric populations of a marine copepod. *Proc. Natl. Acad. Sci. USA* **99**, 12955-12958.
Roff, D. A. (1992). *The Evolution of Life Histories*. New York: Chapman and Hall.
Rosenzweig, F. and Sherlock, G. (2009). Through a glass clearly. Experimental evolution as a window on adaptive genome evolution. In *Experimental Evolution. Concepts, Methods, and Applications of Selection Experiments* (ed. T. Garland, Jr and M. R. Rose), pp. 353-388. Berkeley: University of California Press.
Savageau, M. A. and Sorribas, A. (1989). Constraints among molecular and systemic properties: implications for physiological genetics. *J. Theor. Biol.* **141**, 93-115.
Selander, R. K. (1976). Genetic variation in natural populations. In *Molecular Evolution* (ed. F. J. Ayala), pp. 21-45. Sunderland, MA: Sinauer.
Shirley, B. W. (1996). Flavonoid biosynthesis: "new" functions for an "old" pathway. *Trends Plant Sci.* **1**, 377-382.
Somero, G. N. (1969). Pyruvate kinase varaints of the Alaskan king crab. *Biochem. J.* **114**, 237-241.
Stanley, S. M. and Parsons, P. A. (1981). The response of the cosmopolitan species, *Drosophila melanogaster*, to ecological gradients. *Ann. Ecol. Soc. Australia* **11**, 121-130.
St-Cyr, J., Derome, N. and Bernatchez, L. (2008). The transcriptomics of life history trade-offs in whitefish species pairs (*Coregonus* sp.). *Mol. Ecol.* **17**, 1850-1870.
Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.
Storz, J. F. and Wheat, C. W. (2010). Integrating evolutionary and functional approaches to infer adaptation at specific loci. *Evolution* **64**, 2489-2509.
Streisfeld, M. A. and Rausher, M. D. (2009). Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species. *New Phytol.* **183**, 751-763.
Streisfeld, M. A. and Rausher, M. D. (2010). Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* doi:10.1111/j.1558-5646.2010.01128.x.
Townsend, C. R. and Calow, P. (1981). *Physiological Ecology. An Evolutionary Approach to Resource Use*. Oxford: Blackwell Scientific Publications.
van Delden, W. (1982). The alcohol dehydrogenase polymorphism in *Drosophila*. *Evol. Biol.* **15**, 187-222.
van Strallen, N. M. and Roelofs, D. (2006). *An Introduction to Ecological Genomics*. Oxford: Oxford University Press.
Verrelli, B. C. and Eanes, W. F. (2001a). Clinal variation for amino acid polymorphisms at the *Pgm* locus in *Drosophila melanogaster*. *Genetics* **157**, 1649-1663.
Verrelli, B. C. and Eanes, W. F. (2001b). The functional impact of *Pgm* amino acid polymorphism on glycogen content in *Drosophila melanogaster*. *Genetics* **159**, 201-210.
Watt, W. B. (1991). Biochemistry, physiological ecology, and population-genetics. *Funct. Ecol.* **5**, 145-154.
Watt, W. B. and Dean, A. M. (2000). Molecular -functional studies of adaptive genetic variation in prokaryotes and eukaryotes. *Annu. Rev. Genet.* **34**, 593-622.
Willet, C. S. and Burton, R. S. (2004). Evolution of interacting proteins in the mitochondrial electron transport system in a marine copepod. *Mol. Biol. Evol.* **21**, 443-453.
Wright, K. M. and Rausher, M. D. (2010). The evolution of control and distribution of adaptive mutations in a metabolic pathway. *Genetics* **184**, 483-502.
Zera, A. J. (2005). Intermediary metabolism and life history trade-offs: Lipid metabolism in lines of the wing-polymorphic cricket, *Gryllus firmus*, selected for flight capability vs. early age reproduction. *Integr. Comp. Biol.* **45**, 511-524.
Zera, A. J. and Harshman, L. G. (2001). Physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* **32**, 95-126.
Zera, A. J. and Harshman, L. G. (2009). Laboratory selection studies of life-history physiology in insects. In *Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments* (ed. T. Garland, Jr and M. R. Rose), pp. 217-262. Berkeley, CA: University of California Press.
Zera, A. J. and Harshman, L. G. (2011). Intermediary metabolism and the biochemical-molecular basis of life history variation and trade-offs in two insect models. In *Molecular Mechanisms Underlying Life History Evolution* (ed. T. Flatt and A. Heyland). Oxford: Oxford University Press. In Press.
Zera, A. J. and Zhao, Z. (2003). Life history evolution and the microevolution of intermediary metabolism: activities of lipid-metabolizing enzymes in life-history morphs of a wing-dimorphic cricket. *Evolution* **57**, 568-596.
Zera, A. J. and Zhao, Z. (2004). Effect of a juvenile hormone analogue on lipid metabolism in a wing-polymorphic cricket: Implications for the biochemical basis of the trade-off between reproduction and dispersal. *Biochem. Physiol. Zool.* **77**, 255-266.
Zera, A. J. and Zhao, Z. (2006). Intermediary metabolism and life history trade-offs: differential metabolism of amino acids underlies the dispersal-reproduction trade-off in a wing-polymorphis cricket. *Am. Nat.* **167**, 889-900.
Zera, A. J., Koehn, J. G. and Hall, J. G. (1985). Allozymes and Biochemical Adaptation. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 10 (ed. G. A. Kerkut and L. I. Gilbert), pp. 633-674. Oxford: Pergamon.
Zhao, Z. and Zera, A. J. (2002). Differential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket. *Proc. Natl. Acad. Sci. USA* **99**, 16829-16834.
Zufall, R. A. and Rausher, M. D. (2004). Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* **428**, 847-850.