# Perspectives

## Anecdotal, Historical and Critical Commentaries on Genetics

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# Allozymes in Evolutionary Genetics: Self-Imposed Burden or Extraordinary Tool?

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**THE controversial history of allozyme studies:** Alternative heritable forms of enzymes, differing in charge or shape, have been known since the 1940s; these may be alleles of one gene (allozymes) or products of distinct but related genes (isozymes). LEWONTIN and HUBBY (1966), finding an unexpected bonanza of allozyme variation in Drosophila, re-cast the existing debate about the evolutionary meaning of genetic variation in terms of allozymes. A torrent of like data followed; its interpretation was dominated at first by the notorious "neutralist-selectionist" debate. Population-genetic theory alone proved unable to resolve this debate; pure genetic-statistical analyses lacked power to test deviations from neutrality (EWENS and FELDMAN 1976), and neutralist and selectionist models predicted convergent distributions of allelic/genotypic frequencies (GILLESPIE 1991). Too little *biology* was present in the debate, and studying the impacts of allozymes on biological mechanisms in the wild promised to help. Mechanistic study of allozymes has indeed ensued, and its practitioners are mostly optimistic. LEWONTIN (1991), in contrast, stigmatized allozyme study since 1966 as a "fardel" or frustrating burden. Some others share his skepticism. Such clashing views bespeak varying awareness of what has been found, or else paradigm differences or other communication barriers. Here, I summarize progress in mechanistic allozyme study, critique reservations about it and explore its promise for new research.

**What has been learned from mechanistic study of allozymes?** A thorough review is impossible here. I illustrate points with a subset of well analyzed cases, apologizing to those whose important work is omitted or discussed cursorily. I often cite recent summaries rather than original references.

*Function of allozymes in metabolic context:* Consider a 1-substrate-1-product enzyme-catalyzed reaction described by

$$v = \frac{(V_{\max_f}/K_{m_f})[A] - (V_{\max_r}/K_{m_r})[B]}{1 + [A]/K_{m_f} + [B]/K_{m_r}}$$

where $v$ is net reaction rate, $f$ and $r$ mark parameters of forward and reverse reactions, $[A]$ and $[B]$ are substrates/products, $K_m$s are composite constants which index substrate affinity (but are not strict dissociation constants), and $V_{\max}$, the maximum velocity, is the product of enzyme concentration $[E]$ and catalytic rate constant $k_{cat}$. The ratio $V_{\max}/K_m$ is the limiting pseudo-first-order rate constant as $[A]$ (or $[B]$) decreases. Enzyme stability differences may change $[E]$. Variants in transcriptional or translational regulation, changing $[E]$, may co-occur with allozymes' peptide-specific differences (*e.g.*, LAURIE and STAM 1988); this can mimic variation in $k_{cat}$, but not variation in $K_m$.

How do metabolic effects arise from changes in these allozyme parameters? Metabolic network theory (KACSER and BURNS 1973; EASTERBY 1973; SAVAGEAU and SORRIBAS 1989) is central to a clear answer. Metabolism may be in steady state (all rates in the pathway equal to the system flux rate, metabolite pool sizes unchanging) or transient state (rates and metabolite pools changing). In either case, most ("intervening") steps must evolve high (*not* "excess") catalytic power (= high $V_{\max}/K_m$) if control of steady state flux, or of speed of transient response, is to be focused on a few steps which thus are "rate-limiters" and whose properties may then be refined *coadaptively*. No allozymes have been studied at rate-limiting steps (except for Hb), so high $V_{\max}/K_m$ has been a performance criterion for allozyme studies. $V_{\max}/K_m$ can increase *via* tighter binding, *i.e.*, low $K_m$ (*too* low $K_m$ may be harmful, HOCHACHKA and SOMERO 1973), or by increased $V_{\max}$, through higher $[E]$ or $k_{cat}$ ($V_{\max} = k_{cat}[E]$).

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Thermal stability changes [*E*] *via* effects on enzyme half-life. Location of an enzyme in a "branch point" among pathways may intensify the impact of change in its metabolic parameters. Connection to fitness measures (WATT 1986), mutation-selection balance models (CLARK 1991), etc., promises more evolutionary utility of this theory.

*Mechanistic study of allozymes' evolutionary impact:* Case studies may be thought of in terms of FEDER and WATT's (1992) view of evolution as a recursion of four stages, from the starting genetic makeup of one generation to the same point of the next: a) genotypes → phenotypes, how genetic variants change organisms' "design" (*e.g.*, protein structure, body form, etc.); b) phenotypes → performance, how organismal design supports important activity such as feeding, locomotion, or regulation; c) performance → fitness, how organisms' performance translates through demography into survivorship or fecundity, thence into net fitness; d) fitness → genotypes, how fitnesses set (or fail to set, if genetic drift or inbreeding forestall them) the next generation's genetic makeup. The whole recursion has been traced in the case of human Hb. Some newer case studies are nearly complete, and others are close behind:

• Ten genotypes of the glycolytic enzyme phosphoglucose isomerase (PGI) in lowland Colias butterflies differ strikingly in kinetics and thermal stability, often trading off between these qualities as anticipated by HOCHACHKA and SOMERO (1973). Some but not all heterozygotes are superior in kinetics; PGI genotypes *4/4* and *4/5* are equally kinetically poor and thermally stable compared to "sister" genotypes, thus being neutral with respect to one another even while they differ sharply from others at the same gene. The major $V_{max}/K_m$ advantage of *3/4* over *4/4* genotypes of Colias PGI is reflected, as predicted, in a severalfold advantage of *3/4* over *4/4* in flux response (detected with radioisotope tracers) through Colias' flight muscle glycolysis during flight. Differences among the PGI genotypes in daily flight capacity, predicted from the biochemical differences, were found in extensively replicated field experiments. The flight differences in turn were predicted to translate into genotypic differences in survival, male mating success, and female fecundity; predictions have been tested and confirmed in replicate among seasons, years, populations, and two semi-species. Genotype frequencies, reflecting the fitness component results in genotype-specific fashion, have shown closely similar values across western North America for 36–100$^+$ generations, depending on local demography. More fitness component trials in extreme habitats, then quantitative synthesis of the components into net fitness, will complete analysis of this selection regime [WATT (1992) and references therein].

• LABATE and EANES (1992) have recently found major effects of Drosophila glucose-6-phosphate dehydrogenase (G6PD) allozymes *in vivo*: a 32% difference in pentose shunt flux among genotypes arises from 40% difference in their kinetics.

• In a clinal lactate dehydrogenase (LDH) polymorphism of the fish Fundulus, the heterozygote enzyme's kinetics are more like the cold-specialist homozygote at low temperature (10°) and more like the warm-specialist homozygote at high temperature (40°). At 10°, the kinetic differences between genotypes successfully predict their carriers' erythrocyte ATP/hemoglobin (Hb) ratios, hence Hb $O_2$ loading (ATP being used by fish to modify Hb function), and predicted differences among the genotypes in egg hatch and in swimming speed are experimentally confirmed. At 25°, allozymes' similarity leads to a *lack* of difference in ATP/Hb values. These functional differences have been used successfully to predict survivorship differences among the LDH-B genotypes. A cline of Fundulus' LDH frequencies along the Atlantic coast of North America, from northern near-fixation of the cold-specialist allele to southern fixation of the warm-specialist allele, follows directly from the lower-level analysis [POWERS *et al.* (1991) and references therein].

• Other such cases include Drosophila alcohol dehydrogenase (ADH) (VAN DELDEN 1982; FRERIKSEN *et al.* 1991; LAURIE and STAM 1988), Metridium sea anemones' PGI (ZAMER and HOFFMANN 1989), α-Hb in Peromyscus mice (CHAPPELL and SNYDER 1984), Tigriopus copepods' glutamate-pyruvate transaminase (GPT) (BURTON and FELDMAN 1983), and leucine aminopeptidase (LAP) of Mytilus mussels (KOEHN 1987) [see WATT (1985, 1991) and POWERS *et al.* (1991) for yet others and more detail].

• Major allozyme differences are not universal. There is little kinetic difference at 37° among PGI allozymes of *Escherichia coli*; in turn, these alleles are the same in fitness at 37° in chemostat competition to within *s* (selection coefficient) ≈ 0.002 (DYKHUISEN and HARTL 1983). This result is sometimes said to "oppose" other case studies, but does no such thing: obviously, lack of difference in allozymes' function should yield lack of difference in allozymes' fitness (WATT 1985)!

These results undermine extreme neutralist and selectionist views alike: allozymes' biochemical function differs often *but not always*; non-additive heterozygote intermediacy is most usual, but overdominance also occurs. These functional differences have *specifically predictable* impacts on metabolic and physiological performance, and in turn on diverse fitness components.

**Challenges faced by mechanistic evolutionary study of allozymes:** If the above is so, why is the approach still controversial? Some workers still harbor reservations, whether or not stated in print. Concerns should be addressed, and naiveté requires correction,

but also, mistaken concerns should be identified.

*Null alleles:* LANGLEY *et al.* (1981) studied these in two wild Drosophila samples. Nulls at 25 allozyme loci had frequencies of 0.0–1.2% among 357–912 alleles. Assuming mutation/selection frequency balance, phenotypic effects of the null heterozygotes were estimated, on average, as minimal. How, some ask, can recessiveness of nulls be consistent with findings of strong phenotypic effects of allozyme variants?

As the authors' statistics show, these null frequencies are heterogeneous within, and similarly so between, samples. Of 58 nulls, 41 were at 5 of the 25 loci, while 10 loci had 0 or 1 null. These data provide no meaningful average for heterozygous effect of nulls, yet the question about strong effects of allozymes relies on just such an average. (Also, for the rarer nulls, a frequency estimated from, *e.g.*, one sample of 1/716 and one of 0/436 is likely to be an *over*estimate, *under*estimating heterozygous phenotypic effect.) Next, it is a *non sequitur* to say that if null mutants are recessive at some loci in one taxon, variants at other loci or in other taxa must also be recessive. This study of null variants needs follow-up in terms of differing protein structures or functional roles of loci *vs.* null frequency, but its results do not conflict with evidence of other variants' phenotypic and/or fitness-related effects.

*Metabolic aspects of dominance:* KACSER and BURNS (1981) restated WRIGHT's (1934) argument for a metabolic cause of dominance: an intervening metabolic step working in steady state may have enough catalytic power to be "haplo-sufficient" (two copies of an impaired allele needed to produce major phenotypic damage). Going beyond WRIGHT, they claimed that allozymes should therefore have little phenotypic effect, but this does not follow because:

- Many pathways are not selected to focus control on a few rate-limiting steps, so no one step has enough catalytic power that its mutants are recessive.
- Enzymes' kinetics, stability, and [*E*] will change in pathway evolution only so far as selection dictates (WATT 1986; CLARK and KOEHN 1992). This will often entail functional compromise between mean and extreme conditions. Thus, haplo-sufficiency may often be narrowly limited, such as within a thermal optimum (*cf.* WATT 1991).
- Pathways often operate in transient-state conditions, which are much more demanding and much less likely to allow haplo-sufficiency.

Thus, when dominance occurs, the WRIGHTian mechanism often explains it, but embedding allozymes in metabolic networks does not, *per se*, render their phenotypic effects recessive, nor does it imply that allozymes usually are without metabolic effect.

*Genetic load:* LEWONTIN and HUBBY (1966) posed the problem thus: if balancing selection acted on allozymes at thousands of loci in a population, the cumulative disadvantage of homozygotes might wipe out the population. Besides reduction of this problem by diverse assumptions or selection regimes (*e.g.*, GILLESPIE 1991), the argument does not undermine allozyme studies because we do not find, in one species, thousands of varying allozymes *or* uniform selection on them. Most studied allozymes work in energy processing or biosynthesis; while centrally important, there are only 300–500 such loci in a species. Usually ≤25% of these are polymorphic at once, and the nature and strength of selection varies widely among loci (above). Thus, genetic load arguments do not clash with specific findings of major allozymic effect.

*Linkage disequilibrium:* Effects attributed to allozymes might instead be caused associatively by tightly linked variants of unknown genes. Linkage disequilibrium is unstable to recombination, but special conditions can produce it, so it merits consideration in each study of natural genetic variation. In purely structural-genetic terms, only DNA-sequence-level finding of linkage equilibrium between a selected site and its neighbors can fully test associative alternatives. However, associative alternatives can also be tested with great power on other grounds.

One major associative effect is "hitchhiking" wherein a directionally selected allele is followed in its frequency rise by a neutral allele at a closely linked gene (THOMSON 1977). This might confound apparent differences among allozymes, especially those lacking clear functional cause, but again, recombination opposes it. Neutral variants hitchhike with old, selected variants in a narrow range, *e.g.*, Drosophila ADH accumulates plausibly neutral "silent" variants (which do not change amino acids) only within ≈200 base pairs of the selected site, well within the ADH gene (HUDSON, KREITMAN and AGUADÉ 1987; AQUADRO 1992). Strong selection may extend this range, but asymmetric selection narrows it (ASMUSSEN and CLEGG 1981).

Moreover, any view of allozymes as neutral associates of other genes strongly predicts the *absence* of connections between allozymes' properties and organism-level or fitness differences. Given the diversity of genes and the general eukaryotic absence of linkage among genes controlling a process (save for some multi-gene families), there is minimal chance of correlation between genotypic patterns of even one enzyme property (*e.g.*, $V_{max}/K_m$) at a truly neutral gene and patterns of selection on a linked gene. So, when allozymes' functional differences can predict performance and fitness-related effects in a genotype-specific way, associative hypotheses (*e.g.*, hitchhiking) require additional postulates: (a) tightly linked genotypes, which actually cause observed effects and realistic mechanisms for their action, and (b) mechanistic reasons why the allozymes' differences do not cause the effects predicted from them. Without evidence for

these postulates, associative views of functionally and fitness-distinct allozymes are negated by Ockham's razor: "Do not multiply entities needlessly."

*Complications of pleiotropy or epistasis:* "Fitnesses at one gene vary with fitnesses at others." This does not, as some claim, preclude meaningful study of allozymes. If pleiotropy or epistasis were impenetrable, genetics would be impossible. Allozymes are powerful tools just because they are specific probes of metabolic hierarchies in Darwinian context. Background effects and genetic or phenotypic correlations do occur, and mean effects of allozymes may be complicated by interaction with other variation, but these issues may be analyzed empirically (*e.g.*, CARTER and WATT 1988; WATT 1992).

*Are allozyme studies "adaptationist"?* Naive adaptationism, seeking separate explanation for each "atomized" trait of an organism, deserves critique. But this pitfall can be avoided, *e.g.*, if allozymes alter a trait without altering fitness, then to the extent of the change, the trait's state is not adaptive. *E. coli*'s PGI $K_m$ is not differently adaptive among its allozymes at 37° (DYKHUISEN and HARTL 1983), while Colias' PGI $V_{max}/K_m$ is adaptive with precision down to the 20–30% difference between *3/4* and *3/3* genotypes, which leads to, *e.g.*, major genotypic fecundity effects (WATT 1992). Using allozymes to probe adaptation need not entail adaptationist bias.

*Are allozymes peripheral to modern evolutionary study?* LEWONTIN (*e.g.*, 1980) and others say that adaptation is peripheral to the logic of evolution, which would lessen the utility of allozyme studies. They claim that three propositions are necessary and sufficient for natural selection: (1) phenotypic variation, (2) heritability of the variants and (3) differential reproduction of the variants. But, as is clear from DARWIN (1859; *cf.* BRANDON 1990), *this claim is wrong*: these three propositions, while *necessary* for natural selection, are *sufficient* only for *arbitrary* selection, wherein we do not know the cause of differential reproduction. DARWIN held that natural selection resides in the demographic *results* of differences among heritable variants in suitedness to their environment, *i.e.*, *differences in adaptation.*

KRIMBAS (1984) claimed that this makes evolution "circular" or "tautological." This charge may fit the confused aphorism "survival of the fittest" but it fails against DARWIN's basic concept. The evolutionary recursion is not circular unless causative adaptive and resulting fitness differences have been mistakenly conflated. As for tautology, do not confuse the tautological nature of well defined, logically (or algebraically) true statements, such as DARWIN's argument, with the empirical issue: do these statements, or this argument, rightly describe the world? DARWIN was neither circular nor tautological in posing adaptation as a central *empirical* problem for evolutionary study.

Some question whether allozymes typify traits of most evolutionary interest: complex morphologies or performances, which many expect to be under polygenic control. But allozymes have large fitness-related effects through such complex performances as, *e.g.*, locomotion (Fundulus LDH, Colias PGI), cold stress tolerance (Peromyscus Hb), or osmoregulation (Mytilus LAP, Tigriopus GPT). This also suggests that major fractions of the genetic variance in complex traits may be neither additive nor polygenic, and hence ill described by usual quantitative genetic models.

Others argue that study of adaptation, hence of allozymes, is particularist. If so, it is better to know about specific cases than to know nothing about adaptation; but beyond that, generality is seldom found unless sought. If few generalities about allozymes have yet been made, that does not imply futility of future attempts.

*Is evolution too complex to measure?* One anonymous skeptic, perhaps speaking for others, remarked of mechanistic evolutionary genetics that it " . . . is heuristic, but ignores the true complexity of evolution . . . . " But is this really so? What difficulties could lead to this claim, and are they real or illusory?

Demographic or genetic-transmission subtleties can be accounted for. Subpopulation mixing effects may mimic genotypic survivorship differences, but can be ruled out when population structure is known and allelic covariances can be calculated (WATT 1983). Segregation distortion or assortative mating can be studied during the progeny analysis of mating success testing, as for Colias PGI, where neither effect was found (WATT, CARTER and BLOWER 1985). Genetic drift and inbreeding cause irreproducibility of genotypic differences, or characteristic distortion of genotypic frequency patterns.

Catastrophism is said to preclude evolutionary prediction, but it is not at issue here. A population's extinction by a stochastic hundred-year weather event (*e.g.*, EHRLICH *et al.* 1972) erases its evolutionary history, but our task is to explore what *is* predictable about evolution, not to despair in the face of stochastic complications.

Habitat diversity concerns some workers in relation to possible variation or antagonism of selection pressures, but one may replicate performance or fitness studies across microhabitats; proper field work accounts for this in its designs. Allozymes' effects may indeed be antagonistic, as in red deer whose IDH allozymes reciprocally change female survival and fertility, but this may maintain the variation (PEMBERTON *et al.* 1991). One must evaluate all major, ecologically relevant performances and fitness components before making final conclusions about maintenance of genetic variation, but this may be easier than has been feared. Where organisms' niche structure is well

understood, rigorous experiments can be done with statistical testing against explicit null hypotheses (above; FEDER and WATT 1992).

The mechanistic study of evolution, using allozymes as tools or probes, in no way ignores complexity either of allozymes' phenotypic expressions or of their translation into large, small, or zero fitness differences. Rather, like all other science, it moves by successive refinement toward full understanding of relevant complexity. A quasi-vitalistic reluctance to believe that this is possible will help no one.

**Where can we go from here?** WATT (1985, 1986) and CLARK and KOEHN (1992) stress a bioenergetic focus on allozymes' impacts. More work in this line will be fruitful, *e.g.*, can bioenergetic cost-benefit theory of metabolic evolution evaluate which alternatives of change in $[E]$, $k_{cat}$, and $K_m$ should be favored by selection in specific cases? Of course, this is not the only possible context for allozyme evaluation. Overall adaptation might well be a supervenient (ROSENBERG 1978) "umbrella" under which bioenergetic, mating-system, or other contexts for allozyme evaluation might be co-important.

Nucleic-acid analysis may complement allozyme work and *vice versa* (KREITMAN, SHORROCKS and DYTHAM 1992). DNA-sequence analysis of allozymes, together with coalescence theory, allows inference of selection or its absence, though alone it gives no clue to biological causes (AQUADRO 1992). The combination of these approaches has much to offer, *e.g.*, DNA sequencing easily reveals the amino acid variation underlying allozymes' properties. Conversely, mechanistic study of allozymes gives the biological sources of selection (or its absence) whose statistical correlates may be found by sequencing. Also, sequencing is basic to studying the extent of linkage disequilibrium, $D$, around selected sites in allozymes (above). Complementary functional study of the allozymes can then probe how $D$ varies with the nature or strength of selection.

Further exploration of habitat variation will greatly aid allozyme work, *e.g.*, food supply variation selects on allozymes in Apodemus mice (LEIGH BROWN 1977), and Colias' esterase-D allozymes covary with food plant use, suggesting a role in detoxifying plants' chemical defenses (BURNS 1975). The opportunity for new insight is immense if physiological and behavioral ecology are more used in evolutionary genetics.

Allozymes' mechanisms have not yet been much studied in phylogenetic context, yet they could be. For example, the "adaptation to neutral limits" concept of metabolic evolution (HARTL, DYKHUISEN and DEAN 1985) may apply widely to *E. coli* allozymes, yet it does not hold for eukaryotes studied so far (WATT 1991). What phyletically consistent aspects of these taxa, or of their proteins' evolutionary history, might explain this?

**Final remarks:** Many, but not all, allozymes differ in function. These differences translate *predictably* through metabolic and physiological performance into fitness component differences, eventually leading to net fitness differences. In this work, neutrality is the null model. Where allozymes do not differ, this null model is the mechanistic prediction and has been sustained; where allozymes differ significantly, the null model has been falsified as the mechanistic prediction has been sustained. Thus, these studies are not correlational, but follow the alternative-hypotheses decision strategy of PLATT (1964). Among empirical or *a priori* reservations about such studies, some are mistaken, while others must always be considered but can be tested empirically. None pose general barriers to the probing of evolution with allozymes.

The mechanistic study of allozymes (or other natural variants) offers great power for asking and answering both integrative and specific questions that other approaches have not recognized or resolved. Far from being a self-imposed burden, allozymes' functional and fitness-related diversity affords a extraordinary intellectual tool for experimental, genetically informed study of evolution.

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