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THE ROLE OF ALCOHOL DEHYDROGENASE EVOLUTION IN ALCOHOL ADAPTATION OF DROSOPHILA MELANOGASTER

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Dedication

To my parents, who uprooted their lives for foreign shores so that my siblings and I could dare to define and chase our own American Dreams.

Table of Contents

List of Figures

List of Tables

Acknowledgments

The experiences I have had as a graduate student have been among the most formative and meaningful in my life. I will always be grateful to the Ecology and Evolution program at the University of Chicago for providing me with this opportunity.

It is my belief that scientists--and humans--are at their best when they are concurrently encouraged and challenged, and I am lucky to have been a part of a lab that consistently encouraged and challenged me. For this, Joe deserves a considerable amount of credit—the environment in our lab very much reflects his conviction that such an atmosphere is essential for the personal and intellectual development of young scientists. I have also learned much as a mentee from observing Joe's relentless commitment to and investment in the process—regardless of whether this is the process of cooking Marcella Hazan's bolognese sauce, going through an endurance workout, or producing a scientific manuscript—over the exact nature of the outcome. I could not imagine a better lesson to have taken away from my PhD training than the importance of the process because our craft of doing science is, at its core, not about any one outcome but the ongoing human effort of understanding the world a little better tomorrow than we do today.

I am grateful for the guidance I received from my committee of Allan Drummond, Vinny Lynch, Marcus Kronforst, Ilya Ruvinsky, and Marty Kreitman. They have not only aided in improving my science but have continuously inspired me with their own curiosities about the biological world and intricacies therein. Having Marty on my committee was a particular privilege because the subject of his PhD dissertation was the same as the subject of mine, and I got to hear about how evolutionary genetics was done not only before the genomic age but even before the age of thermocyclers. The personal and intellectual generosity Marty showed me as I went through my project was most encouraging and formative. I aspire to someday be able to do the same for the graduate students I work with.

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Chapter 1: Introduction

In this thesis, I use new techniques in functional molecular evolution to test long-held adaptive hypotheses concerning the evolutionary forces and biological mechanisms that have produced intraspecific variation and interspecific divergence at alcohol dehydrogenase locus of *Drosophila melanogaster*. In this survey, I review the relevant conceptual background for this work. Specifically, I 1) review a brief history of the concept of adaptation and its study in evolutionary genetics, including historical and current challenges, 2) highlight how these limitations are now being overcome in modern studies of adaptation and the challenges that persist, and 3) provide a description of the *alcohol dehydrogenase* (ADH) system of *D. melanogaster*, the central subject of this this thesis.

1.1 Adaptation in the development of evolutionary genetics

Adaptation is the process of evolutionary change through which organisms become suited to their environments, and how this process unfolds is the among longest studied and widely debated topics in evolutionary biology [1]. While adaptive features are sometimes viewed as the *de facto* outcomes of "Darwinian" evolution by natural selection [2,3], the evolution of adaptive features was actually the first major scientific challenge to the explanatory power of evolution by natural selection, as proposed by Darwin [4]. The challenge to Darwin's idea was not that living organisms were related through ancestry--among his critics were his close colleagues, Huxley and Galton, who staunchly defended the idea of all living things being related by evolution--but that selection on small differences over evolutionary timescales was not a sufficient explanation for how organisms acquired highly distinct, adaptive features in different environments.

Darwin had proposed that adaptation happens through gradual directional evolution of traits that vary in a continuous way, and it was clear that such continuous variation abounded in populations of organisms. However, analysis and interpretations of breeding data showed that parents who were exceptional for a continuous trait produced offspring with a lower value for that trait. Discovery of this phenomenon--regression to the mean--brought into question the explanatory power of Darwin's proposed mechanism for adaptive evolution: How could such a

slow, gradual process lead to the diversity of features that fit different organisms to vastly different environments when there was such propensity for "all exceptional stock to revert to that mediocrity, whence the majority of their ancestors originally sprang"? [5]. An alternative proposed mechanism was that adaptations evolved through large, discontinuous changes which were inherited reliably, that "the progress of evolution is not a smooth and uniform progression, but one that proceeds by jerks, through successive 'sports',...each in its turn being favored by Natural Selection." [6]. Following the rediscovery of Mendel, these "sports" would become known as Mendelian traits, and whether adaptation occurred primarily through inheritance of Darwinian or Mendelian traits would remain the first long, contentious debate in evolutionary biology [4]. The discovery that genes could describe patterns of inheritance for both Darwinian and Mendelian traits would lead to the birth of population genetics and articulation of a conceptual framework that possessed the necessary components to make predictions about trait evolution and how they might be caused by genes of various numbers and effect sizes. Today, those of us who study evolutionary genetics and adaptation often motivate our work with the question, "Does evolution proceed through few, large steps or many small steps?" A more appropriate sentiment may be that this isn't just a question in evolutionary genetics but, more so than any other, this is the question that founded the field.

The Modern Synthesis [7], as the research of this period in the early 20th century has come to be known, provided a theoretical framework from which predictions could be made about how genetic changes cause adaptation and, conversely, how adaptations shape genetic variation in populations and species. Understanding the genetic causes and consequences of adaptation to different environments therefore required connecting the variation in putatively adaptive phenotypes to variation in underlying genotypes across populations and/or species. However, no precise methods existed to characterize genetic variation, making the task of linking genotype to phenotype impossible. The Modern Synthesis had provided the theoretical foundation for the field, but the same theoretical machinery could be used to reach very different predictions about the genetics of adaptation, as seen in the contrasting views of Wright and Fisher or Dobzhansky

and Muller [1,4]. The only thing preventing these hypotheses from being decisively tested--it was thought--was the inability to get measurements about the amount of genetic variability in populations [1].

Technological advances eventually overcame this challenge and direct characterization of genetic variation became ubiquitous, first because of gel electrophoresis and subsequently because of DNA sequencing. Between the publication of Lewontin and Hubby's original measurement of genetic variability in 1966 [8] and publication of the aptly titled continuation "Still more genetic variability in natural populations" [9] in 1973, the field of evolutionary genetics had embraced measurements of genetic variation as a central, if not *de facto*, mode of inquiry. This methodological commitment has continued since in spectacular fashion. Genetic variation could now be measured and the field realigned itself to measure it, but what did such measurements reveal about adaptation? There was ample variation in populations but most of it could not be ascribed to functional or fitness differences, making it impossible to discern whether genetic variation existed because allelic combinations were beneficial (balancing selection) or because positive selection had not yet finished establishing a best, "wildtype" variant in that population [1,10]. Perhaps most alarmingly, the data best supported a new emerging theory that was more mathematically elegant and had a simple, jarring prediction at its core: most genetic variation within and between species was neutral [11,12]. Motoo Kimura's Neutral Theory changed the question from what type of adaptive evolution leads to genetic differences to whether adaptive evolution really leads to much genetic differentiation at all, fundamentally challenging the presumptive biological importance of most sampled genetic variation between populations and species inhabiting different environments [1,13–17]. Inferences of adaptive genetic variation, therefore, required ruling out this widely predictive hypothesis of neutral evolution. The most decisive way to do so was by characterizing how specific genetic variants affect organismal functions and fitness-components in biologically relevant environments [1]. However, the field--once hampered by the technological inability of measuring genetic variation--became constrained by the commitment to exclusively measure it.

Lewontin, upon reflecting on the effect of the revolution he launched in evolutionary biology, thought many aspects of the unquestioned focus on measuring genetic variation was a millstone, particularly for understanding the genetics of adaptation which necessarily required integrating different levels of biology [18]. His primary concern was that a large-scale reorientation of the field towards measuring genetic variation coincided with a concerning reorientation of the field away from everything else:

> *The immense outpouring of data on genetic variation has also been a millstone around our necks. Its first effect was a considerable depauperization of the diversity of empirical work in evolutionary genetics. Within a few years experiments on fitness variation in natural and laboratory populations, selection experiments on morphological and physiological traits, studies of developmental regulation and flexibility in an evolutionary context, work on chromosomal variation, studies of segregation distortion in natural populations-all of the rich diversity of evolutionary genetic investigation-nearly disappeared from the literature of our subject as one investigator after another discovered the joys of electrophoresis. But the problems raised by those earlier studies have not been solved. They have only disappeared from our collective consciousness.*
> *(Lewontin 1991)*

A goal remained to understand the genetic basis of adaptations but with a greatly reduced focus on understanding the phenotypes and their putative adaptive consequences. These criticisms, originally raised about gel electrophoresis, remained equally valid in the genomic era as the just-so stories of adaptation that emerged to explain ever-greater amounts of information just took on a different form [2,19,20].

1.2 The integrative approach in studies of adaptation

Lewontin was prescient in his articulation of the problem of studying adaptation solely through phenotype or genotype and ignoring the chain of causality that connects genotype, phenotype, and fitness in environments. Research in modern evolutionary genetics has also now become aware of these limitations [2,19,21], and population genetics research has demonstrated the difficulty of discerning genetic changes underlying adaptations from genomic data alone due to the myriad of evolutionary processes and historical events that have shaped modern genomes. Over the last decade or so, a renewed commitment to empirical programs that explicitly test links

between variation at different biological levels has been seen in the field [22], and important findings have begun to emerge from many different parts of evolutionary genetics: from studies of fitness variation in nature [23–34] and laboratory populations [35–38], of directional selection on organismal traits [39], development [40–45], chromosomal variation [46–48], and segregation distortion in natural populations [49–51]. The value of an integrative research program aimed at elucidating causal links across biological levels appears not to have disappeared from our collective consciousness after all.

Commitment to an experimental program in evolutionary genetics has been central to recent advances in our understanding of how organisms have adapted to different habitats. Here, I review studies that serve as exemplars for studying biological adaptations and which highlight why it is necessary to test, and to continue to question, all of the causal links specified above in developing an understanding of how historical and ongoing adaptation happens, and the challenges that continue to exist in making decisive adaptive inferences.

The level of rigor in these studies has come from coupling a commitment to natural history of organisms with specification and testing specific causal links between variation at the levels of genotype, molecular and developmental phenotypes, and organismal performance with respect to environmental factors. The key insights that have emerged from these studies are due to the level of nuance and detail taken into account, and they highlight how a reductionist approach can have great value for discoveries that might be more general in nature. I then briefly review the *Drosophila melanogaster* alcohol dehydrogenase enzyme system, the subject matter for my thesis work of testing hypotheses of adaptive protein evolution, within and between species.

1.2.1 Genetic basis of repeated morphological evolution in freshwater sticklebacks

Threespine sticklebacks (*Gasterosteus aculeatus*) have been studied as a natural system for over a century and over the last two decades have emerged as one of the richest systems for studying the genetic basis of adaptive evolution [52,53]. Migratory marine sticklebacks colonized newly formed freshwater environments following the last ice age, and in the subse-

quent have 10,000-15,000 years have repeatedly evolved numerous differences in morphology, physiology, and behavior across independent, globally-distributed freshwater populations [54]. Freshwater and marine populations differ considerably in their morphology, physiology, and behavior and were mistakenly classified as different species when they were first described [52]. However, they are reproductively compatible and therefore amenable to techniques for genetic mapping. The major phenotypic difference that has been studied between marine and freshwater sticklebacks is pelvic morphology: marine sticklebacks have an armored pelvic morphology consisting of ~30 bony lateral plates and a bony pelvic girdle, whereas this feature is absent or nearly absent across freshwater sticklebacks [54,55]. The consistent presence/absence of these features between marine and freshwater populations suggested that different forms were adaptive in different habitats; physiological studies had shown that sticklebacks that lack the armor developed faster and had greater lipid stores, whereas stickleback that had the armor were less prone to predation in some habitats [31].

Genetic experiments mapped that the presence of bony lateral plates and pelvic girdle to regions containing the Ectodysplasin gene (Eda) and a tissue-specific enhancer of pituitary homeobox transcription factor 1 (Pitx1), respectively. Together these loci were sufficient to explain over two-thirds of the observed variation in the pelvic armor of the fish. Evolutionary sequence analysis also showed that regions containing these two genes were among the most differentiated between marine and freshwater stickleback, and patterns of sequence variation near the derived alleles were consistent with some aspects of recent positive selection. Through a series of manipulative experiments over a decade, the authors showed a single nucleotide mutation in an Eda enhancer disrupts gene expression through the Wnt pathway and causes the loss of later plates [56,57], and *cis*-regulatory deletion in a Pitx1 enhancer causes loss of the pelvic girdle [58]. Both of these mutations are found in multiple freshwater populations. To further test if the if the Eda variant found in freshwaters populations conferred a selective benefit in freshwater environments, the authors simulated historical colonizations. They released populations of sticklebacks that were Eda heterozygotes into four freshwater ponds and monitored how the frequency of the

different pelvic morphs and of the Eda alleles changed over the course of a year. Consistent with their predictions, the frequency of the freshwater Eda variant was significantly higher a year later in the F_1 generation than it was in the parental generation in all four ponds, whereas the frequency of "control" satellite markers dispersed at other parts of the genome showed no consistent trend. Taken together, the authors were able to identify genetic variants through experimental and population genetic mapping approaches and then causally link the candidate adaptive variants to disruptions in gene expression that resulted in the putatively adaptive morphological feature. They were subsequently able to demonstrate through field experiments that both the morphological feature and its underlying genetic variant responded in a way consistent with selection when placed in the natural environment.

The work in the stickleback system connects variation in genotype to variation in development, morphology, and survival in a relevant environment. It is a methodological exemplar of rigorously studying adaptation. Yet, it also highlights how all adaptive (and conversely, neutral) explanations rely on certain assumptions. In this case, it was the premise that the loss of pelvic armoring was the adaptive trait--justifiable on the basis of its repeated appearance in freshwater populations--in freshwater habitats because the costs of its production outweighed the benefits it provided with respect to predation. But what if this was not the trait under direct selection? The stickleback researchers have continued to identify and genetically characterize other traits that vary between freshwater and marine populations. A remarkable finding is that freshwater populations have twice as many teeth as their marine counterparts [59]. This phenotype is caused by a *cis*-regulatory difference that changes BMP4 expression in the late-juvenile stage, and it raises the possibility of an additional or alternative adaptive explanation: the loss of pelvic armor may be a byproduct of changes calcium allocation as they changed from being prey in oceans to predators in freshwater ponds. This is, of course, is a speculative hypothesis. But because of the reductionist approach taken in the study of sticklebacks, it is a testable hypothesis. As technology improves and allows the making of precise transgenics of Pitx1, Eda, BMP4, and other identified loci, it will be possible to isolate the individual and combined effects of these loci on freshwater

survival. Therein lies the value of characterizing traits at the genetic, molecular, and organismal levels, regardless of whether they ultimately end up being adaptive: it allows the use of the scientific method to discern between different plausible accounts of evolutionary history.

1.2.2 Genetics and molecular mechanisms of high altitude adaptation in deer mice

The deer mice (*Peromyscus maniculatus*), one of the most widely distributed rodents in North America [60], has also emerged as a powerful model system for studying adaptation. Some populations of deer mice--like sticklebacks--have undergone rapid morphological evolution, most notably in coat color, following recent geological events [23,61–63]. A different adaptive trait that varies across populations of deer mice is the capacity to survive at vastly different altitudes, and detailed studies of the genetic and molecular mechanisms that contribute to variation in this trait have yielded important insight into the number and molecular nature of genetic cases involved in adaptive diversity.

Deer mice that are native to high altitudes of the Rocky Mountains are capable maintaining higher organismal performance in response to hypoxia than deer mice found nearby in the Great Plains [60,64]. Hemoglobin (Hb), the primary molecule in mammalian oxygen transport, shows considerable genetic variation across deer mice, and population genetic sampling of hemoglobin diversity between high and low altitude populations showed that different variants--separated by many mutations--segregated at high frequencies in the different locales [65]. These data suggest a clear genetic hypothesis for adaptation in this system: genetic changes in hemoglobin that increased the affinity of Hb for oxygen allowed some organisms to maintain higher physiological function in hypoxia-like conditions and therefore were favored at high altitudes, where oxygen levels are lower. Natarajan et al. (2013) tested this hypothesis and found evidence to corroborate it: the Hb variant that was most common at high altitudes (Hb-H) had higher oxygen affinity than Hb variant that was most common at low latitudes (Hb-L) [27]. After getting this result, Natarajan et al. noted that the genetic differences between Hb-H/Hb-L molecules are confined to three distinct regions and proceeded to characterize the individual and

context-dependent effects of each of these regions on oxygen affinity. They found that mutations were highly context dependent (epistatic). In Hb-H, for example, changing only one of the three parts not only decreased the molecule's affinity for oxygen but did so to magnitudes below that of the lowland variants, and in Hb-L, introducing regions from Hb-H caused a decrease--instead of the expected increase--in oxygen affinity. The detailed experimental approach here reveals that the adaptive phenotype--appropriate oxygen affinity for environment--likely results from selection on a specific combination of variants and not on individual variants themselves. Further, it provides a clear demonstration of intramolecular epistasis affecting numerous polymorphic sites and being a cause for the maintenance of genetic diversity between populations.

Why is it necessary to mechanistically test hypotheses concerning adaptive genetic variation when the adaptive explanation--changes in hemoglobin affect altitude adaptation--is so intuitively obvious? Other research from the same lab provides a clear example. After conducting the research above, the researchers asked whether the genetic and molecular mechanisms they observed were also at play in other altitudinally differentiated populations of deer mice. They sought out and characterized Hb alleles that segregate at high and low altitude across deer mice populations in California, in which many of the polymorphisms seen in the Rocky Mountains/ Great Plains regions also segregate but do so alongside other population specific variants [26]. They again characterized the most common Hb variants that segregate at high and low altitudes, but they found, surprisingly, that there was no evidence for functional divergence between the low and high altitude forms. Thus, genetic variation in Hb provides a compelling explanation for altitude adaptation in one locale but not in another. There are numerous reasons for why this may be so: 1) The California specific polymorphisms may epistatically mask the effects of the mutations that are effective in Rocky Mountains and Great Plains. 2) Altitude may not be as strong of a selective pressure in California as it is in the Rocky Mountains. 3) Other physiological mechanisms for dealing with hypoxia-like conditions may have diverged between California populations. 4) The more continuous spatial gradient in California might permit sufficient gene flow between populations such that local adaptation does not happen. A deeper understanding of the

genetic, molecular, and physiological mechanisms that underlie altitude adaptation will require consideration of these hypotheses.

1.3 The *Drosophila* alcohol dehydrogenase system:

The alcohol dehydrogenase enzyme (ADH) of *D. melanogaster* is one of the most well-studied proteins in biology [66,67]. Parallel interests in ADH for its sequence variation, molecular properties, physiological effects, and natural distribution led it to become a study system in numerous disciplines and one of the first systems where specific adaptive hypotheses could be articulated about causal links between various biological levels. Differences in ethanol tolerance between *D. melanogaster* and other species, as well as differences between geographically distinct populations of *D. melanogaster*, made ADH a particular appealing candidate gene for studying adaptation.

The importance of ADH as a historically important model system is hard to overstate: It was one of the first proteins in which electrophoretic variation was characterized and in which electrophoretic variants corresponded to functional differences [68]; it was one of the first enzymes in which variants were found to be highly differentiated among geographically separated populations and correlated with an ecologically important trait [69]. It was the first gene from *D. melanogaster* to be cloned [70], the first gene for which population level DNA sequence variation was characterized [71], the first system to which now ubiquitous population genetic tests of neutrality and selection were applied [72–74], and also the first gene where difficulties of interpreting DNA sequence structure became apparent [73]. The patterns of ADH sequence divergence, enzyme function, relationship to ethanol-related physiology, and geographical distribution strongly suggested some kind of selection was acting on the gene, either between species and/or between populations of *D. melanogaster* [18]. A decisive experimental account of what, if anything, was selected for never materialized. Clear hypotheses emerged and subsequently became textbook examples of adaptation, but they--like adaptive hypotheses of previous decades--were not directly testable at the time due to technological limitations in engineering precise transgenic strains and characterizing pure, controlled quantities of protein. However, decisively supported

explanations regarding whether ADH evolved adaptively between and/or within species did not emerge, likely due to many experimental limitations at the time. The level of precision needed to separate the biochemical effects of protein coding differences from those of regulatory variants-- at the *Adh* locus and elsewhere in the genome-- [75,76] did not exist. Nor could precise transgenic animals be engineered so that the effect of variation from this locus could be studied at the level of physiology and fitness in different environments, as necessary to decisively test predictions entailed in different adaptive hypotheses. Today, those technical limitations can be overcome and consequently the specific predictions entailed within those hypotheses can be tested.

In this dissertation work, I test long-standing hypotheses about the genetic and functional mechanisms through which ADH evolved and was involved in adaptation of *D. melanogaster* to high-alcohol environments. In my first chapter, I use ancestral sequence reconstruction, biochemical characterization of enzymes, and physiological characterization of animals harboring ancestral ADH variants to test whether historical evolution in the *D. melanogaster* ADH increased activity and led to higher organismal ethanol tolerance. In my second chapter, I use biochemical and physiological assays to test whether balancing selection acting on a stability-activity maintains different ADH variants across latitudinal clines. In my third chapter, I provide a review of studies that have used ancestral sequence reconstruction to characterize the number and molecular nature of genetic changes through which molecular specificity has historically evolved and highlight common themes emerging from such studies regarding protein functional evolution.

Chapter 2: Experimental test and refutation of a classic case of molecular adaptation in *Drosophila melanogaster*

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2.1 Summary

Identifying the genetic basis for adaptive differences between species requires explicit tests of historical hypotheses concerning the effect of past changes in gene sequence on molecular function, organismal phenotype, and fitness. Here we address this challenge by combining ancestral protein reconstruction with biochemical experiments and physiological analysis of transgenic animals carrying ancestral genes. We tested a widely held hypothesis of molecular adaptation – that changes in the alcohol dehydrogenase (ADH) protein along the lineage leading to *Drosophila melanogaster* increased the enzyme's catalytic activity and thereby contributed to the species' ethanol tolerance and adaptation to its ethanol-rich ecological niche. Our experiments strongly refute the predictions of the adaptive ADH hypothesis and caution against accepting intuitively appealing accounts of historical molecular adaptation based on correlative evidence. The experimental strategy we employed can be used in the future to decisively test other adaptive hypotheses and the claims they entail about past biological causality.

2.2 Introduction

A central goal of molecular evolutionary biology is to identify the genes and biological mechanisms that mediated historical adaptation. Rigorously testing hypotheses in this area has been a major challenge. Many studies infer past selection from statistical signatures in genes involved in biological processes that might have suited species to their environments [77–80], but sequence signatures of selection can be forged by chance or demographic processes, and predicting how changes in gene sequence affect phenotypes and fitness is challenging [2,18,19,81]. Compelling evidence for molecular adaptation therefore requires formulating and testing ex-

plicit hypotheses about the causal links between specific evolutionary changes in gene sequence and resulting changes in molecular function, organismal phenotype, and fitness [1,2,18,19,82]. Advances in genetic mapping, experimental studies of molecular function, and transgenic engineering have allowed hypotheses of molecular adaptation between recently diverged populations to be tested with increasing rigor [28,58,62,65,83,84]. But hypotheses about adaptive divergence between species or at higher taxonomic levels are explicitly historical, and testing them directly requires measuring the effect of genetic changes that occurred in specific evolutionary lineages in the distant past on phenotype and fitness. Here we address this challenge by combining ancestral protein reconstruction [85] with biochemical experiments and physiological analysis of transgenic animals carrying ancestral genes.

We applied this approach to a longstanding hypothesis of molecular adaptation – that changes in the alcohol dehydrogenase (ADH) protein of the fruit fly *Drosophila melanogaster* increased the enzyme's catalytic activity and thereby contributed to the species' adaptation to its ethanol-rich ecological niche [82,86–88]. This hypothesis was articulated decades ago [82,86] and became widely accepted [82,88–90] based on several observations that were consistent with it but did not directly address the putative causal links among historical changes in protein sequence, function, and fitness. First, *D. melanogaster* evolved to colonize ethanol-rich habitats in rotting fruit after it split from its sister species *D. simulans* some 2 to 4 mya [91,92]. Second, fractionated cell extracts from *D. melanogaster* catalyze alcohol turnover more rapidly than those from *D. simulans [76,86,93]*. Third, the first-ever application of the McDonald-Kreitman (MK) test detected an excess of nonsynonymous substitutions in an alignment of the ADH coding sequences of *D. melanogaster* and closely related species [72], which was interpreted as evidence for adaptive evolution driving the divergence of the ADH protein between *D. melanogaster* and *D. simulans [16,82,89,94]*. These observations were integrated into an account by which adaptation to ethanol-rich habitats was driven by selection on the ADH protein sequence for increased catalytic activity. Other factors – particularly increases in the expression level of ADH [76,95–97], changes at other genetic loci [98–101], and within-species polymorphisms [75,100,101]– are

also likely to have contributed to ethanol adaptation in *D. melanogaster*, but they are independent of and cannot explain the selection signature on the ADH enzyme's protein-coding sequence found in the MK test.

We focused on the hypothesis of adaptive ADH protein evolution because it is widely accepted based on correlated forms of variation in extant species and because it is particularly amenable to testing using the experimental approaches of ancestral reconstruction, biochemical characterization, and engineering of transgenic organisms. The ADH adaptive hypothesis entails specific, testable predictions about how genetic changes that occurred in the ADH protein sequence during the historical divergence of *D. melanogaster* affect the phenotype at several levels—molecular function (catalytic turnover of ethanol by pure ADH protein), physiology (ethanol catabolism in the animal's tissues), and fitness components (survival in the presence of ethanol) (**Figure 2.1A**). We tested these predictions by reconstructing the ADH protein from the last common ancestor of *D. melanogaster* and *D. simulans* (AncMS) and experimentally characterizing how changes in ADH sequence along the *D. melanogaster* lineage affected ADH function, physiology, and fitness.

2.3 Results

2.3.1 Reconstruction and synthesis of ancestral and extant *Drosophila* alcohol dehydrogenases

We generated a large alignment of ADH sequences, determined the best-fit evolutionary model, inferred the maximum likelihood phylogeny, and calculated the posterior probability distribution of amino acid states at key ancestral nodes. Specifically, we synthesized coding sequences for the maximum a posteriori sequence of AncMS, which was inferred with high confidence and only one ambiguously reconstructed amino acid (**Figure 2.1B**, **Appendix 1, Figure A1**), and for an alternative version of AncMS (Alt-AncMS), which contained the other plausible state at the ambiguous site and is identical to *D. simulans* ADH. We also characterized the inferred ancestral *D. melanogaster* ADH, the amino acid sequence of which is identical to that of the "Slow" allele present in extant populations, which is known to be older than other ADH

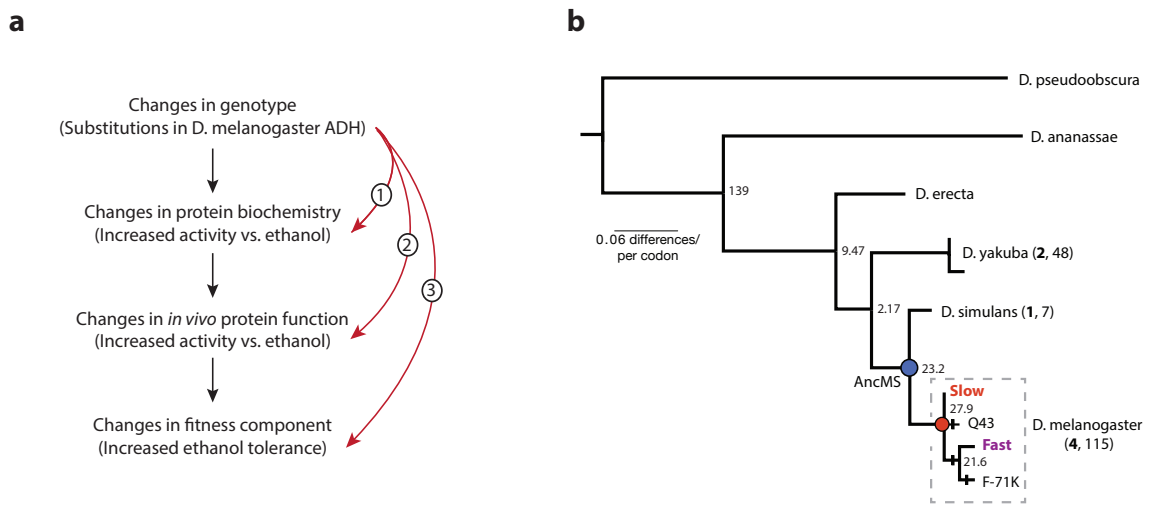

Figure 2.1: Predictions of the classic hypothesis of ADH adaptive evolution. A) Hypotheses of molecular adaptation entail putative causal links (black arrows) between evolutionary change in genes and effects on molecular function, organismal phenotype, and fitness. In parentheses are the specific links that comprise the classic hypothesis of ADH adaptive evolution in *Drosophila melanogaster*. Red arrows represent testable predictions entailed by this hypothesis. We tested the predicted effects on enzyme activity (link 1) and on physiology (link 2) and fitness components (link 3) using purified reconstructed ancestral ADH proteins and animals carrying reconstructed ancestral alleles. B) Maximum-likelihood phylogeny of ADH protein sequences. The classic ADH adaptive hypothesis predicts functional divergence on the branch connecting the last common ancestor of *D. melanogaster* and *D. simulans* (AncMS, blue) to the ancestral *D. melanogaster* ADH allele (red). Polymorphic ADH protein variants are shown, including the Slow allele (which is identical in sequence to the ancestral *D. melanogaster* sequence, red circle) and the derived Fast allele (purple). The number of distinct segregating protein alleles (bold) followed by the number of sampled alleles is displayed in parentheses for species with available polymorphism data. Node labels show statistical support as approximate likelihood ratios. Scale bar for branch lengths shows the expected number of substitutions/codon.

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variants [102]. The adaptive ADH hypothesis predicts differences in ethanol catalysis between the AncMS ADH and the ancestral *D. melanogaster* ADH. In addition, we characterized the "Fast" allele, a more recently derived ADH variant, to determine whether the assays we used are sensitive enough to detect previously identified phenotypic differences thought to be of selective importance in some natural populations of *D. melanogaster* [18,101,103].

2.3.2 Historical sequence changes that occurred in D. melanogaster ADH do not alter biochemical properties

We first tested the prediction that genetic change in the ADH protein along the *D. melan-*

ogaster lineage caused divergence in *in vitro* biochemical function. Unlike the studies performed decades ago on fractionated homogenates from present-day flies, we were able to directly measure the functional effects of specific historical changes in protein sequence by using heterologously expressed ancestral proteins and improved methods for purification and quantification. We found, contrary to the prediction of the adaptive ADH hypothesis, that both the maximal catalytic turnover rate of ethanol per enzyme molecule (k_{cat}) and the Michaelis-Menten constant (K_m) – a measure of the enzyme's performance when substrate concentration is limiting – were indistinguishable among AncMS, *D. melanogaster*, and *D. simulans* ADH proteins (**Figure 2.2**). The assay was sensitive enough to detect the Fast allele's expected increase in ADH cata-

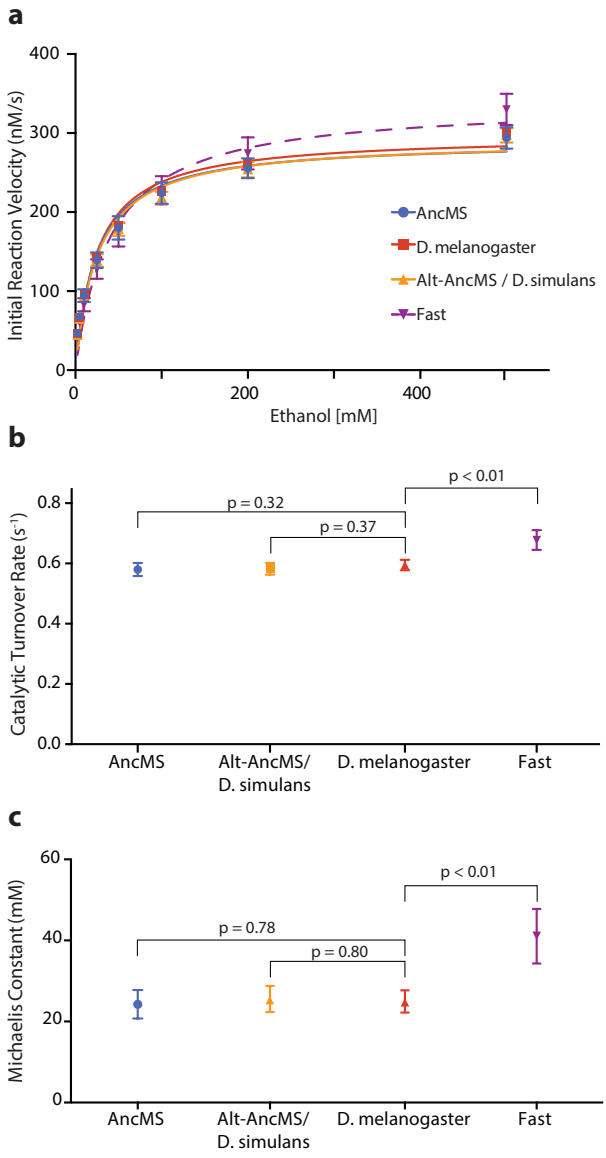

Figure 2.2: Effects of ADH sequence divergence on the activity of purified enzymes. *In vitro* assays of bacterially expressed protein show no divergence in catalytic properties of ADH between the ancestral form (AncMS) and the *D. melanogaster* protein. Alternative reconstructed sequence (Alt-AncMS, identical in sequence to *D. simulans* ADH) and the derived Fast allele are also shown. A) Michaelis-Menten plot of initial reaction velocity across ethanol concentrations. Points show mean and 95% confidence interval of nine measurements at each concentration. B) Estimated maximum catalytic turnover rate (k_{cat}) and C) Michaelis-Menten constant (K_m) for each allele. Points and error bars show the estimated parameter and 95% confidence interval calculated by nonlinear regression from the data in panel A. P-values for differences are from likelihood ratio tests comparing a global model in which a single value of the parameter of interest is estimated from the data for both genotypes vs. a free model with separately estimated parameters.

lytic function. When enzyme activity was measured using isopropanol – a higher-activity ADH substrate not thought to be ecologically important – we again observed no difference between the ancestral and *D. melanogaster* alleles, whereas the Fast allele displayed enhanced activity. (**Appendix 1, Figure A2; Table A1**).

2.3.3 Historical sequence changes that occurred in D. melanogaster ADH do not alter *in vivo* enzyme activity or organismal ethanol tolerance

Second, the adaptive ADH hypothesis predicts that sequence evolution in *D. melanogaster* should cause differences in ethanol catabolism *in vivo*. Differences in solubility, translational efficiency or accuracy, post-translational modifications, stability, or presence of other cellular co-factors could cause ADH proteins to behave differently when produced *in vivo*. To test whether divergence of the ADH protein sequence caused biochemical differences in ethanol catabolism *in vivo*, we genetically transformed Adh-null *D. melanogaster* with ancestral or extant ADH alleles that differed only in their amino acid sequences. We raised these transgenic flies to adulthood and measured catabolism of ethanol by homogenates from each genotype under maximum velocity conditions. Contrary to the adaptive hypothesis' prediction, homogenates from flies expressing the *D. melanogaster* ADH allele did not have higher rates of ethanol turnover than AncMS or Alt-AncMS. Again, the derived Fast allele was associated with significantly faster ethanol turnover (**Figure 2.3A**).

Finally, the adaptive ADH hypothesis predicts that divergence of the ADH protein along the *D. melanogaster* lineage should enhance fitness by increasing survival in ethanol-rich environments. At both larval and adult stages, flies carrying AncMS, Alt-AncMS, and *D. melanogaster* ADH alleles had statistically indistinguishable ethanol tolerance, measured as the dose of ethanol causing a 50% probability of death (i.e., the LD_{50}) (**Figures 2.3B, C**). In contrast, the Fast allele conferred higher ethanol tolerance in larvae (**Figures 2.3B**). Thus, divergence of the ADH protein sequence along the *D. melanogaster* lineage had no detectable effect on survival in the presence of ethanol.

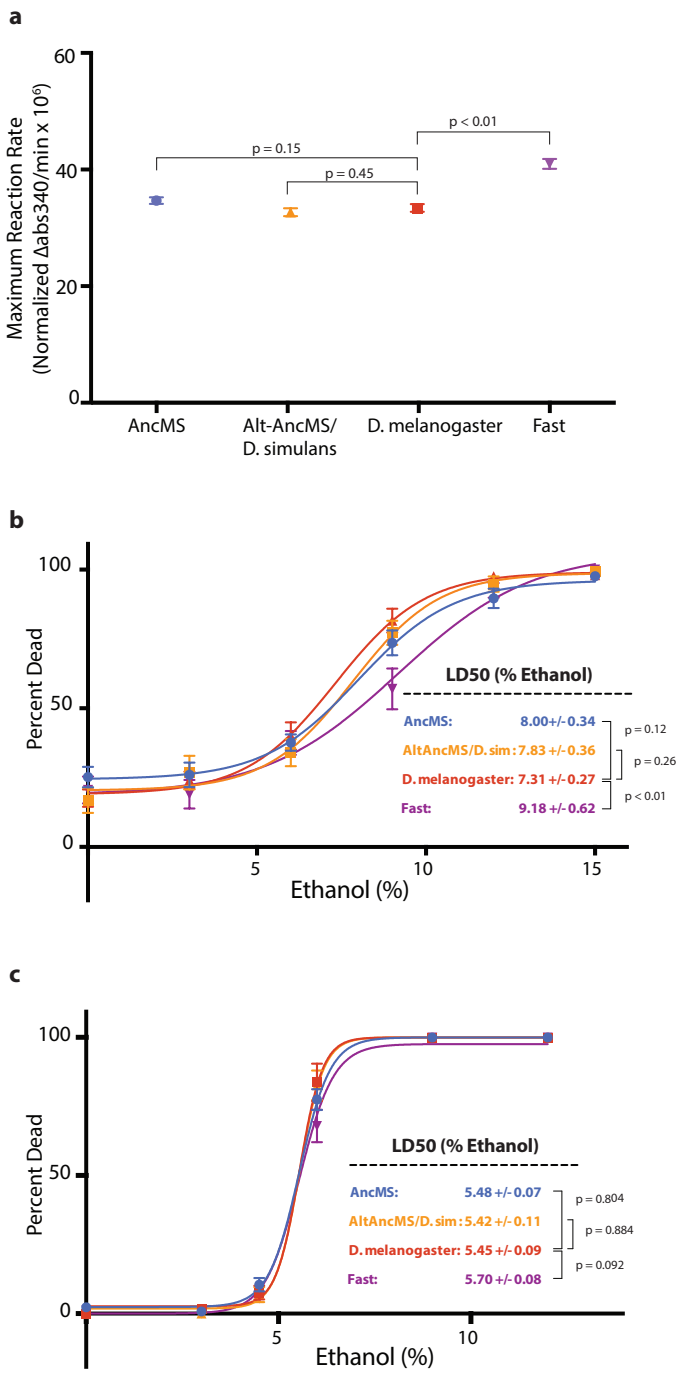

Figure 2.3: Effects of ADH sequence divergence on ethanol catabolism and fitness in transgenic flies. Adh-null *D. melanogaster* were genetically transformed to express coding sequences of ancestral or extant ADH proteins; genotypes were otherwise identical. A) Ancestral and *D. melanogaster* ADH alleles do not confer differences in ADH catabolism. Animals of each transgenic genotype were homogenized and the soluble fraction assayed for ethanol turnover rate under saturating substrate conditions. The graph shows maximal reaction rate normalized per mg of total protein content of the homogenate. Points and error bars show the mean and SEM of 30 replicate homogenates. P-values are from t-tests for differences in means for genotype pairs of interest (see Methods). B,C) Effect of ADH genotype on ethanol tolerance. Transgenic larvae (B) and adults (C) were assayed for survival in the presence of increasing ethanol concentration. Points and error bars show mean and SEM for 8 to 10 replicate groups (for larvae) or 4-8 replicate groups (for adults) at each dose. Estimated LD50 (ethanol dose causing a 50% probability of death, adjusted for baseline mortality) is given for each genotype with 95% confidence intervals. P-values for comparisons are from likelihood ratio tests comparing a global model in which a single LD50 is fit to the pooled data from both genotypes to a free model with an independent LD50 for each genotype.

2.3.4 The sequence signature of adaptive protein evolution on the *Drosophila* phylogeny is spuriously associated with evolution on the *D. melanogaster* lineage.

These experiments indicate that historical substitutions in the ADH coding sequence along the *D. melanogaster* lineage cause none of the predicted effects on biochemical function, physiology, or fitness components, refuting the widely-held hypothesis of adaptive ADH divergence. Why then

did the original statistical analysis of the ADH coding sequence suggest positive selection[28]? We considered two possibilities. First, the inference of positive selection might have been a stochastic error due to sparse sampling of polymorphisms; we therefore repeated the McDonald-Kreitman (MK) test using a much-expanded contemporary dataset [104], with greater sampling of polymorphism. We found that the signature remained (**Appendix 1**, **Table A2**). Second, the signature of selection might come from lineages other than *D. melanogaster*, because the MK test in its standard form does not apportion sequence changes onto phylogenetic lineages. We therefore conducted a polarized MK test on the expanded dataset by assigning substitutions to specific branches on the phylogeny; we also conducted a standard MK test but with individual species removed. We found no signature of positive selection on the *D. melanogaster* lineage, and removing *D. melanogaster* from the analysis did not affect the MK result (**Figure 2.4A, Appendix 1**, **Figure A3, Table A2**). In fact, there is only one nonsynonymous substitution along the putatively adaptive *D. melanogaster* branch, at N-terminal residue 1 of the mature protein, in a solvent-exposed loop far from the active site (**Figure 2.4B)**. Rather, the detected signature

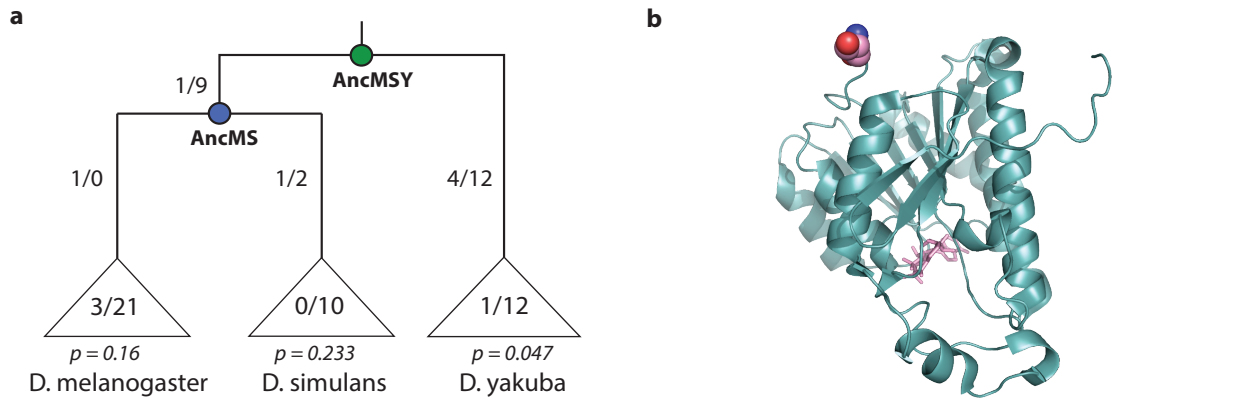

Figure 2.4: Sequence evolution on the phylogeny of *D. melanogaster* and closely related species.
A) The signature of adaptive evolution in the ADH coding sequence is not caused by substitutions on the *D. melanogaster* lineage. Substitutions were assigned to specific lineages based on maximum likelihood ancestral reconstructions at each node. Labels show nonsynonymous/synonymous substitutions (on branches) and polymorphisms (in triangles). McDonald-Kreitman tests were conducted separately for each species, using substitutions that occurred on the branch leading to each species and polymorphisms in extant populations of that species. P-values show significance of the test for each species. B) The nonsynonymous substitution that occurred during the divergence of *D. melanogaster* from AncMS. The structure of *D. melanogaster* ADH (PDB 1MG5) is shown as cyan cartoon. Spheres show the Ala1Ser substitution near the protein's N-terminus, far from the active site and substrate (pink sticks).

of selection comes primarily from the lineage leading to *D. yakuba,* where we observed a marginally significant excess of nonsynonymous divergence ($p = 0.047$). Whether this result reflects adaptive evolution, relaxed constraint, sampling error, or drift is unknown. *D. yakuba's* ethanol tolerance is no different from that of closely related species and is lower than that of *D. melanogaster [98,105]*.

2.4 Discussion

A strength of the ADH adaptive hypothesis was that it entailed specific predictions about the effects of genetic divergence along the lineage leading to *D. melanogaster* on protein function, organismal phenotype, and components of fitness. Ancestral sequence reconstruction, engineering of transgenic organisms, and biochemical/physiological assays allowed us to test these predictions directly. Our experiments show that none of these predictions hold.

We did not test any of the innumerable other hypotheses that have been or could be proposed concerning fruit fly adaptation to rotting fruit or alcohol dehydrogenase evolution. For example, evidence suggests that *D. melanogaster*'s increased ethanol tolerance may have evolved because of substitutions at other loci [98,99] or in regulatory regions of *Adh [97,98]*, and it is possible that these changes were positively selected. The single amino acid replacement that occurred along the *D. melanogaster* lineage could have affected functions other than ethanol catabolism – such as breakdown of other substrates – and, if it did, these changes may or may not have increased fitness. For these or any other claims of molecular adaptation, further work would be required to formulate a specific adaptive hypothesis and test its causal predictions.

Our experiments also provide information relevant to a different question – how ADH alleles segregating in present-day *D. melanogaster* populations affect fitness. Our data show that the amino acid polymorphism distinguishing the Fast and Slow alleles do confer measurable differences in ethanol catabolism and ethanol tolerance, even in the absence of other linked and functionally important genetic variants [106]. These results provide an initial corroboration of the hypothesis that the Fast/Slow polymorphism in protein sequence is biologically and ecologically

important in present-day populations [74,101,103,107]. The differences in ethanol tolerance that we observed between transgenic flies carrying Fast and Slow coding alleles, however, are small relative to the large range of ethanol tolerances observed among *D. melanogaster [98],* and the amino acid changes in ADH are not sufficient to explain the extent of variation in ethanol tolerance within this species. Further work is required to propose and test specific causal hypotheses about why these alleles are distributed in clines that correlate with latitude [101,107] and why the polymorphism is balanced in *D. melanogaster [74]*.

The strategy we employed may be useful in efforts to increase the rigor of scientific inferences about adaptation [2,19]. A hypothesis of molecular adaptation is a conjecture that particular changes in genotype during history caused particular evolutionary changes in phenotype that enhanced fitness: a signature of selection in a gene sequence may suggest such a hypothesis but cannot test it. The case of ADH shows that the existence of variation between present-day species in genotype, phenotype, and fitness is also insufficient to test a hypothesis of molecular adaptation, even if it is consistent with it, because covariation alone does not demonstrate the hypothesized causal links among these forms of variation or the historical direction of the evolutionary trajectory that produced them. We were able to directly test the predictions of the adaptive ADH hypothesis by combining ancestral protein reconstruction with biochemical studies of recombinant proteins and transgenic engineering of organisms carrying ancestral alleles. A similar approach could be applied to test many other adaptive hypotheses. This strategy has some limitations: not all ancestral sequences can be reconstructed with confidence, only some phenotypes can be characterized experimentally, and laboratory experiments cannot detect all fitness differences. Further, manipulative experiments can never account for the full range of genomic and environmental variables that affect organisms' biology and evolution. Some notions concerning adaptation will therefore remain difficult to study rigorously. Nevertheless, because of technical and conceptual advances, it should now be possible to experimentally assess the causal predictions of many previously untested or weakly tested hypotheses of historical molecular adaptation, allowing them to be corroborated or, like the classic hypothesis of ADH divergence

in *D. melanogaster*, decisively refuted.

2.5 Methods

Phylogenetics and inference of ancestral sequences. Coding DNA sequences for species in the *D. melanogaster* group, as well as from outgroup species *D. pseudoobscura,* were obtained from Genbank, the DPGP2 consortium [104], and Daniel Matute (University of North Carolina). DNA coding sequences of alleles that differed in their protein sequence were aligned using MUSCLE, and a maximum likelihood phylogeny was inferred in PhyML (v. 3.0) using the best-fit parameters and model TrN+G, as determined by AIC (jModelTest software, v. 2.1.7). Ancestral sequence reconstruction was performed using the maximum likelihood method [108] in PAML software (v. 4.8); sequences were analyzed using the GY94 general codon model with model=0, nssites =1 3x4 codon frequencies, and transition/transversion ratio inferred were from the data. The posterior probability distribution of ancestral states at each site was analyzed at nodes corresponding to the last common ancestor of *D. melanogaster* and *D. simulans (*AncMS) and to the last common ancestor of all *D. melanogaster* alleles. Sites were considered ambiguously reconstructed if two or more states had posterior probability >0.2.

Synthesis, expression, and purification of ADH alleles. For bacterial expression of ADH proteins, the coding sequence of the *D. melanogaster* Slow ADH allele was generated by de novo synthesis (GenScript, Parsipanny, NJ). Coding sequences for other alleles were generated by site-directed mutagenesis of the Slow sequence using the QuickChange method (Stratagene, San Diego, CA) and verified by Sanger sequencing. Coding sequences were cloned into pLIC-MBP plasmids to yield fusion proteins with maltose binding protein and an N-terminal hexahistidine tag. Plasmids were verified by sequencing and transformed into *E. coli* BL21(DE3) Rosetta cells. Cells were grown at 37 C, and expression was induced using 1mM isopropyl B-D-thiogalactoside (IPTG) at OD_{600} = 0.6. Cells were harvested by centrifugation after reaching OD_{600} of 1.5 - 1.8 and then frozen. To purify proteins, cells were lysed using B-PER, lysozyme, and DNAse I. Lysate was passed over a nickel-affinity HIS-trap chromatography column to isolate the MBP/ADH protein. The MBP tag was removed by treating with sample tobacco etch

virus (TEV) protease overnight and then purifying using HisTrap and cation columns. Purified ADH proteins were flash frozen in 10% glycerol solutions and stored at -80 until they were ready to be characterized.

Transgenic organisms. To make *D. melanogaster* flies carrying *Adh* alleles that differ only in the amino acid sequences they code for, we used the site-specific fC31-attP system. First, a 7.8kb segment containing the *Adh*-Slow allele and all known cis-regulatory elements was amplified from genomic DNA of *D. melanogaster* strain Canton-S; this segment contains the entire transcriptional unit including ADH and ADHR coding sequences with their introns, plus untranscribed sequence extending 2.9 and 1.6 kb in the 5' and 3' directions, respectively. This PCR product was gel-extracted and ligated into the AscI and NotI sites of the attB vector pS3aG. This vector was then modified to facilitate further cloning of alternate ADH coding sequences by amplifying the vector by PCR with primers that incorporate BspQ1 restriction sites at the boundaries of the coding region; digesting with BspQ1 removes the coding region and allows replacement with a new coding region. Variant coding sequences were produced by PCR-amplifying the Canton-S Slow allele ADH coding sequence (including its introns) in overlapping pieces using primers containing the desired nonsynonymous mutations, assembling the fragments in pGem-T-Easy (Promega, Fitchburg, WI) using Gibson Assembly Master Mix (New England Biolabs, Ipswitch, MA), producing a full-length amplicon of this variant coding sequence (with introns) by PCR, and then inserting the amplicon into the vector containing the flanking sequences by Gibson asesmbly. Sequences of amplicons at each stage and of all final vectors were verified by Sanger sequencing. This process produced transformation vectors that coded for the ancestral *D. melanogaster*, AncMS, Alt-AncMS, and Fast protein alleles but were otherwise identical. Plasmid DNA for injection was prepared using the Nucleobond Xtra Midi Plus EF kit (Macherey-Nagel, Düren, Germany) and adjusted to 1 mg/ml.

Constructs were injected into the inbred recipient strain "pf86"[49], which is null for *Adh* and contains the attP landing site *ZH-86Fb* and the phiC31 integrase (genotype y[1] M{vas-int.Dm}ZH-2A w[*]; Adh[fn6] cn[1]; M{3xP3-RFP.attP}ZH-86Fb). Injected G0 flies were backcrossed to

the pf86 strain. F1 transformants carried the w+ allele and were identifiable by eye color. These transformants were crossed to w- sibs, and transformant lines were made homozygous. Lines were tested for correct insertions via PCR. At least two independent transformation strains were generated for each *Adh* genotype.

Enzyme assays. For enzymes purified from bacteria, activity of 500nM ADH enzyme were characterized in a solution containing 1mM Nicotinamide adenine dinucleotide (NAD), 50mm sodium phosphate pH 7.6, and ethanol or isopropanol concentrations of 2.5, 5, 10, 25, 50, 100, 200, and 500mM, with three replicate reactions at each concentration. The rate of reaction was measured every 30 seconds by monitoring absorbance at 340nm, which corresponds to the concentration of NADH, a byproduct of ethanol oxidation. The first five observations for each reaction were used to estimate the initial velocity. This procedure was repeated three times on separate days. Data were pooled and the best-fit values of K_m and k_{cat} were estimated using the Michaelis-Menten nonlinear regression function in GraphPad Prism 7.0. Differences between parameters associated with different ADH genotypes were assessed using the extra sum of squares F-test as implemented in GraphPad Prism 7.0, which uses a likelihood ratio test to compare the likelihood of a model with a globally fitted parameter (k_{cat} or K_m) to one in which the parameter is fit individually to each genotype.

ADH enzyme activity from transgenic flies was measured from crude fly homogenates using the "manual grinders" protocol described in Loehlin and Carroll (2016) [109]. For each transformation strain, we propagated three replicate cultures (broods), each of which was initiated by placing five females and two males in a vial of yeast-free food to lay eggs for two to three days. After pupation and eclosion, all 0- to 24-hours-old adult males were transferred to a fresh vial; this procedure was repeated on five separate days, yielding 15 replicate vials of flies for each transformation strain. When males in a vial reached four days of age, two flies were collected and homogenized using a Potter-Elvehjem homogenizer in 400 mL of 0.1M sodium phosphate buffer, pH 8.6, then centrifuged at 21000 x g for 5 minutes at 4 C; supernatant from each vial's homogenate was split among three replicate enzyme assays and three replicate protein concentra-

tion assays. Assays for ADH enzyme activity and protein concentration (Quant-IT protein assay, Thermo-Fisher, Waltham, MA) were performed as described in Loehlin and Carroll (2016) [109]. Maximal reaction velocity (V_{max}) was measured using ethanol and NAD+ concentrations more than twice those needed to generate maximum rates. The V_{max} of each vial's homogenate was estimated as the mean of the three velocity measurements divided by the mean of the three protein quantity measurements.

Data from the 15 replicate homogenates of each transformation strain were pooled for analysis. V_{max} did not differ significantly between the transformation strains within any genotype (**Appendix 1**, **Figure A4.B, Table A3**), so data from strains of each genotype were pooled for further analysis, yielding 30 replicate Vmax estimates per genotype. Unpaired t-tests were used to test the hypotheses that V_{max} of homogenates from flies carrying the ancestral *D. melanogaster* ADH differed from a) AncMS, b) Alt-AncMS, and c) the ecologically relevant Fast allele. Each t-test represents an independent hypothesis, so we did not correct for multiple testing; however, using a Bonferroni correction did not change the significance ($p<0.05$) of any comparison. Analysis by ANOVA and Dunnett's test for multiple comparisons also found no difference between *D. melanogaster* and AncMS or Alt-AncMS, but a significant difference between Fast and *D. melanogaster* (**Appendix 1**, **Figure A4.C**).

Ethanol survival assays. For each ADH genotype, four to five replicate pools of 25 larvae from each of two independent transformation strains were characterized for survival at each of six ethanol concentrations. Beginning at the 2nd/3rd larval instar transition, 150 individuals in each population were divided into equally sized groups and reared on food containing 0, 3, 6, 9, 12, or 15 percent ethanol. Ethanol-supplemented food was prepared by adding ethanol to a standard molasses-cornmeal Drosophila food to obtain the appropriate percentage ethanol in the total volume of food. To minimize the loss of ethanol due to evaporation, ethanol was added when the food had cooled as long as possible and to less than 60C prior to pouring the food into vials. Vials were plugged immediately after pouring and stored in an 11C refrigerator for no more than three days.

The fraction of individuals surviving to eclosion in each dose group was measured, and the relationship between ethanol concentration and proportion not surviving for each genotype was assessed by fitting a Boltzmann sigmoidal model using nonlinear regression in GraphPad Prism 7.0. The LD_{50} (the concentration at which 50% of individuals are expected to die) was estimated for each genotype from eight to ten replicate pools of larvae at each concentration of ethanol. Significant differences in LD_{50} estimates among genotype pairs were assessed using an extra sum of squares F-test to compare the likelihoods of a constrained model with a single LD_{50} parameter fit to the data from both genotypes to that of a free model with independent LD_{50}s for each genotype.

Adult ethanol tolerance was assayed by placing 25, two to four day old adult flies in vials with Whatman paper containing 1 mL of 3% sucrose solution with either 0, 3, 4.5, 6, 9, or 12 percent ethanol and measuring the fraction surviving after 48 hours. Replication and data analyses were as described for larval tolerance assays, except only one transformation strain per genotype was used.

McDonald-Kreitman tests. The McDonald-Kreitman test [72] was applied to an alignment of sequences from *D. melanogaster*, *D. simulans*, and *D. yakuba*. Analyses were restricted to these three species, as in the original study, because they are the only ones in the *melanogaster* group for which recently collected polymorphism data are available. The alignment included all sequences used in the original study, along with recent polymorphism data provided by the DPGP2 consortium[29] and Daniel Matute (University of North Carolina). We excluded variants sampled only once because rare segregating variants are known to compromise the efficacy of the MK test [110]. For lineage-specific MK tests, we counted the number of nonsynonymous and synonymous divergences that occurred along a branch between reconstructed ancestral alleles, as well as the number of extant polymorphisms of each type within the species descending from that branch.

Data availability. The new sequence data used in this analysis is accessible on GenBank at

KX976486-KX976521. All of the other sequence data is available as a part of the DPGP2 from the Drosophila Genome Nexus (www.johnpool.net/genomes.html). Plasmids, primers, and cell lines used in this study are available from the authors upon request.

Chapter 3: Balancing selection via a stability-activity tradeoff does not maintain clinal variation in the alcohol dehydrogenase enzyme of D. melanogaster

3.1 Summary

A major goal in evolutionary biology is to understand how and why genetic variation is maintained in natural populations. The *alcohol dehydrogenase* (ADH) enzyme of *D. melanogaster* is a well-studied system where variation has been hypothesized to be maintained by balancing selection via a selective trade-off between thermostability and activity. Two major variants of of the Adh gene, named Fast and Slow, segregate worldwide and are distributed along latitudinal clines on several continents; it is widely thought that the Fast allele is favored at high latitudes because it breaks alcohol down faster, whereas the Slow allele is favored at low latitudes because it is more stable when exposed to high temperatures. Here, we use biochemical and physiological assays of precisely engineered genetic variants to test this hypothesis in vitro and in vivo. The Fast protein, as predicted, has higher catalytic activity and increases larval ethanol tolerance, but there is no evidence of a temperature-mediated tradeoff in biochemical or physiological assays. Polymorphisms in the regulatory region of the Fast haplotype increase ethanol tolerance in adults, also at all temperatures tested. Moreover, analysis of a large sequence dataset reveals no signature of balancing selection in the *Adh* gene. These results refute the hypothesis that balancing selection acting on a tradeoff between stability and activity has caused long-term maintenance or the clinal distribution of the Fast and Slow allele classes. Our findings suggest an alternative hypothesis that the Fast allele is favored by selection in high-latitude environments but segregates neutrally at lower latitudes.

3.2 Introduction

A major goal in evolutionary biology is to understand the evolutionary forces and the biological phenomenon that maintain genetic variation within and across populations. Multiple

variants may be maintained by balancing selection – such as heterozygote advantage, frequency-dependent selection, or selection that is spatially or temporally heterogeneous – or by other forces, such as mutation and and migration. Distinguishing among these possibilities remains challenging because the effects of these and other evolutionary factors on sequence patterns can produce similar patterns. One way to address this challenge is through experiments that test specific hypotheses concerning the phenotypic and fitness effects of genetic variation in relevant environments [24,28–30,62,111,112].

The *Drosophila* Adh gene is a classic model system in which genetic variation is thought to be maintained by balancing selection[1,74,113–117]. D. melanogaster larvae and adults use ethanol-rich rotting fruit as a key dietary resource and have uniquely high ethanol tolerance in their species group. The ADH enzyme catalyzes the first step of the ethanol detoxification pathway and is a major determinant of ethanol tolerance in the species [118] . Two forms of *Adh*, encoding enzymes that were named Fast and Slow upon their discovery because of their migration speed on electrophoretic gels, segregate in natural populations [68]. The two allele classes differ by a single amino acid (lysine or threonine at site 192 in Slow and Fast, respectively) and by numerous nucleotide differences at synonymous and noncoding sites. This genetic variation has been hypothesized to be maintained by balancing selection because of a tradeoff between catalytic activity and thermostability caused by the K192T polymorphism [119–124].

This hypothesis was formulated based on several observations: 1) The Fast and Slow genotypes are distributed along stable latitudinal clines in Australia and North America, with Fast being more frequent at high latitudes, where temperatures tend to be cooler, and nearly absent at lower, hotter latitudes [101,107,125]. This geographic distribution mirrors the pattern of phenotypic variation, with *Drosophila* populations from high-latitude regions being generally more ethanol tolerant than low-latitude populations [98,126]. Moreover, the Fast haplotype increases in frequency in the presence of high ethanol concentrations in some laboratory selection experiments [115,127] 2) Lysate from flies with the Fast-genotype has greater catalytic activity against ethanol that lysate from Slow-genotype flies (Vigue and Johnson 1973), but at

higher temperatures, this order is reversed because of putative denaturation of the Fast enzyme. [120–124,128,129] 3) The first papers to apply now-ubiquitous population genetics tests for balancing selection found that patterns of sequence diversity near the K192T polymorphism were consistent with some predictions of balancing selection [73,74,130]. 4) Studies of numerous other enzymes and species found that thermostable enzyme variants are often more frequent in hotter environments, and less stable variants predominate in cooler environments [131–133], and an intrinsic tradeoff was thought to exist between stability and activity, putatively because excess stability may interfere with the structural flexibility required for fast catalysis ([131–135], but also see [136–138]).

These findings are consistent with the balancing selection/activity-stability tradeoff hypothesis, but the precise relationships between the amino polymorphism, temperature-sensitive effects on biochemical activity, and fitness in the presence of ethanol remain unknown. Further, some studies have produced results not predicted by the hypothesis [86,98,102,139–146]. A potential reason for the conflicting results is that ethanol turnover, ethanol tolerance, and heat stress response are affected by many loci -- and by noncoding polymorphisms in regulatory regions of the *Adh* locus, as well -- and some of these are also known to vary geographically [103,106,147–151]. Consequently, the effects of the K192T and other *Adh* polymorphisms on stability, function, and fitness have not been isolated. Here, we precisely engineer genetic variants and characterize their biochemical and fitness-related properties *in vitro* and *in vivo* to test the specific predictions of the activity-stability balancing selection hypothesis for the maintenance of the Fast/Slow alleles.

3.3 Results

3.3.1 The K192 and T192 enzymes do not have different stabilities or temperature-dependence of activity

The hypothesis of an activity-stability tradeoff predicts that the K192T polymorphism should change the thermal stability of the enzyme – specifically that T192, which confers higher

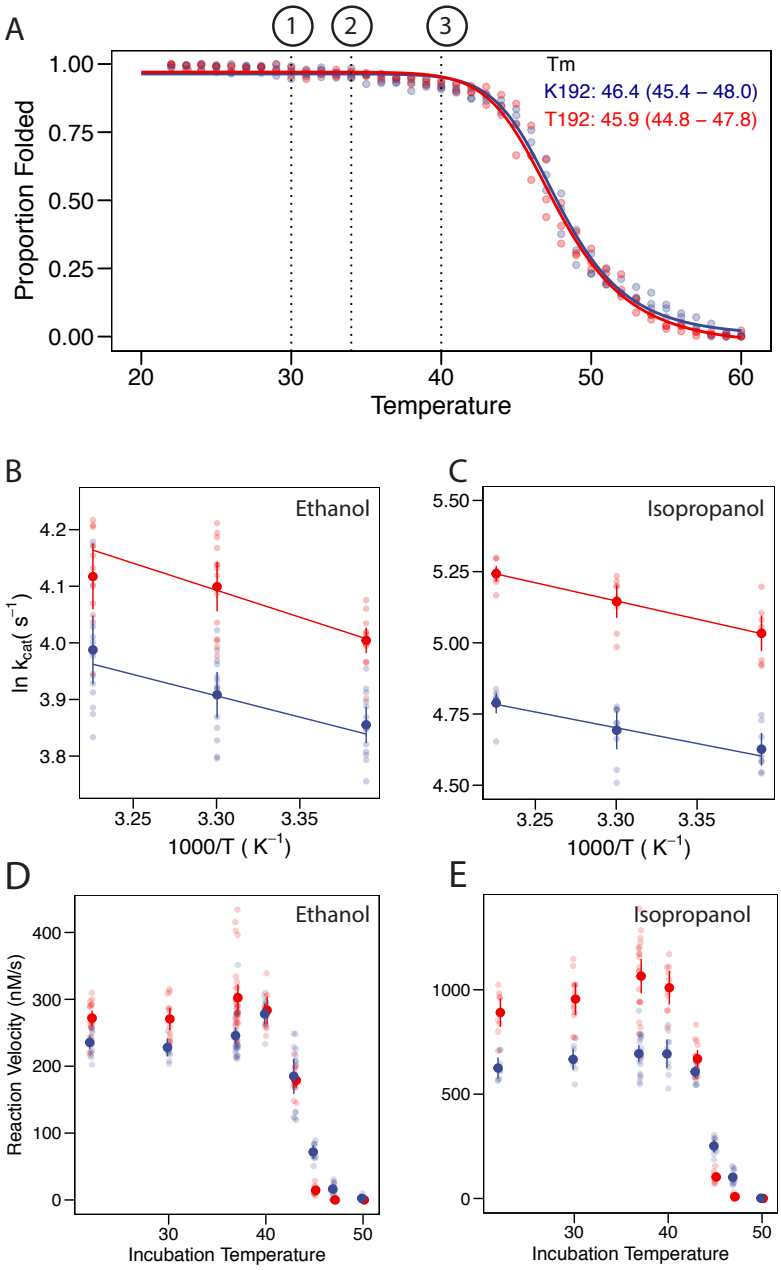

Figure 3.1: The effect of the K192T polymorphism on enzyme thermal stability and temperature-dependent activity.
Measurements of temperature stability and temperature-dependent activity with concentration matched K192 and T192 enzymes show no temperature-mediated tradeoff. A) Circular dichroism measurements of the proportion of folded protein across a temperature gradient. Temperature at which 50% of the secondary structure is lost (T_m) is listed with 95% CI. Dashed lines correspond to temperatures at which the following organismal phenotypes occur: 1) Induction of heat-shock response, male sterility (29-30) 2) Death following chronic exposure (34-35) 3) Rapid death/ incapacitance (40). B&C) Arrhenius plots show enzyme reaction rates at different temperatures. The slopes of reaction rate across temperature are not significantly different between K192 and T192 (ethanol: $p = 0.46$, isopropanol $p = 0.56$). D&E) Enzyme reaction rates following incubation for 1 hour at different temperatures. Light circles correspond to replicates. Dark circles and error bars show the mean and 95% CI at each concentration.

activity, should reduce stability. To test this prediction, we synthesized ADH coding sequences that varied only at the K192T polymorphism and used a bacterial protein expression system to express and purify the two variants. We then used circular dichroism spectrometry (CD) to monitor the loss of secondary structure as temperature was increased. Contrary to predictions, we found that the melting temperature (T_m)—the point at which 50% of the secondary structure is lost—of the K192 and T192 enzymes are statistically indistinguishable (Figure 3.1A). Both proteins begin to unfold at about 42º, well above the chronic temperatures at which D. melanogaster heat shock response is induced [152], males become sterile [153], lethality occurs (Appendix 2, Figure A2.2A), and even short-term exposure incapacitates flies (Appendix 2, Figure A2.2B).

The temperature-mediated trade-off hypothesis also predicts that T192, although it has faster catalytic activity at low temperatures, should have slower activity than K192 at higher temperatures. To test this prediction, we used a spectrophotometric assay to measure ADH activity of controlled quantities of purified enzyme at reaction temperatures from 22º to 37º. Contrary to prediction, T192 has higher maximal activity per unit enzyme (k_{cat}) at all temperatures tested (Figure 3.1B, 3.1C). We also examined whether the two variants differ in their resistance to short-term increases in temperature by incubating each protein at temperatures ranging from 22º to 50º for 1 hour and then measuring catalytic activity at 22º. T192 again displayed higher activity at all temperatures below the incapacitating temperature of 40º; above this temperature, the activity of both variants rapidly declines *in vitro* (Figure 3.1D, E). The same patterns are observed when different alcohols are used. Collectively, these experiments show that the T192 amino acid confers greater activity but, contrary to predictions of the tradeoff hypothesis, there is no evidence that this increase comes at a cost in terms of thermal stability or activity at high temperatures.

3.3.2 The Fast allele increases ethanol tolerance without any temperature-associated costs

It is possible that the T192 variant might cause deleterious temperature-dependent effects on ethanol metabolism or reductions in fitness that are not apparent using *in vitro* assays of catalytic activity. For example, the cellular environment *in vivo* may be different, or physiolog-

ical/fitness effects may be mediated by mechanisms other than a difference in maximal ethanol turnover rate. To isolate the effect of the K192T polymorphism on survival and development in ethanol-rich environments, we engineered transgenic flies that differ solely by this one amino acid by introducing a single coding change into the coding sequence of a natural "Slow" haplotype (Canton-S) and using the phiC31-integrase system to integrate each transgene into the same genomic location in an Adh-null injection strain.

The tradeoff hypothesis predicts that the T192 strain should have higher survivorship on ethanol relative to K192 strains at low temperatures, but lower survivorship at high temperatures. We first tested this hypothesis at temperatures within the normal physiological range of *D. melanogaster*. For each strain, we measured survivorship of approximately 4000 larvae raised at variable ethanol concentrations and temperatures and calculated the ethanol dose at which half of flies die (LD_{50}). At 22º, the LD50s of K192 and T192 strains are not significantly different (Fig. 2A), and there is no statistical effect of genotype on mortality (Appendix 2, Table A2.1A). At 27º, mortality of both strains increases, but the T192 larvae are more resistant to ethanol than K192 larvae (Appendix 2, Table A2.1B), with a significantly higher LD_{50} (Figure 3.2B). These results are inconsistent with the temperature tradeoff hypothesis, which predicts the opposite relationship.

Next, we tested whether the K192T polymorphism affects survivorship in a temperature-dependent fashion when flies are exposed to ethanol as adults rather than during development. For each strain, we measured survivorship of approximately 2000 adult flies of each sex at variable ethanol concentrations and temperatures. There is no difference between the LD50s of K192 and T192 strains, and there is no significant effect of these genotypes on survivorship at either 22º or 27º (Figure 3.2C, D). Both genotypes are more ethanol tolerant at lower temperature, and males are more tolerant than females, but under no conditions tested do K192 flies show higher survivorship than T192 flies (Figure 3.2E).

Another possibility is that T192 might have deleterious effects at temperatures beyond the normal physiological range, which induce the heat shock response in *D. melanogaster*. We

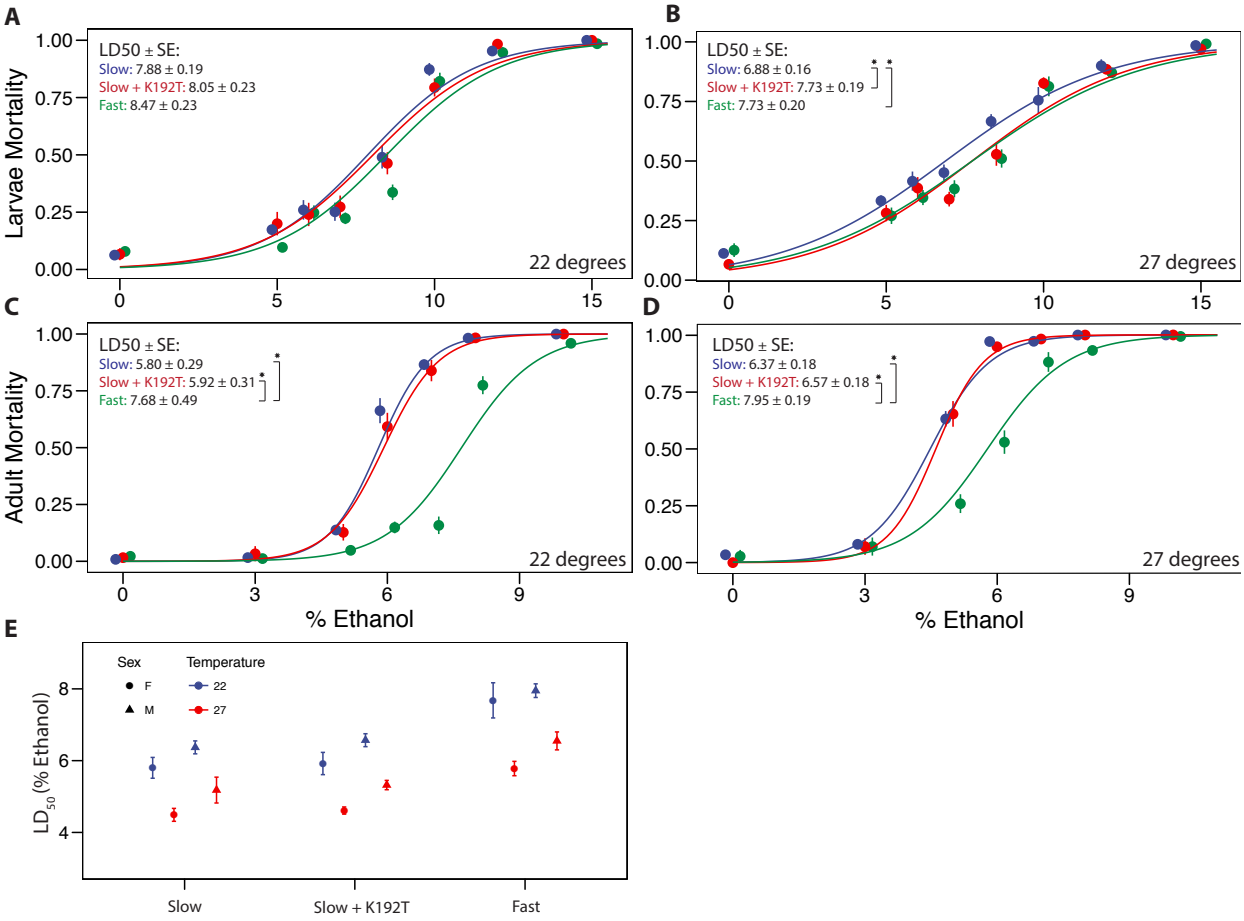

Figure 3.2: Effect of ADH genotype and temperature on ethanol tolerance.
Adh-null *D. melanogaster* strains were transformed with a natural Slow haplotype allele, the Slow haplotype with only the K192T mutation, or a natural Fast haplotype containing the K192T mutation and linked polymorphisms. The T192 amino acid, alone or in the Fast haplotype, increases ethanol tolerance without a temperature-associated cost. Transgenic larvae (A&B) and adults (B,C, & E) were assayed for survival in the presence of increasing ethanol concentrations at different temperatures. Points and error bars show the mean and standard error of the mean for mortality of larvae and of female adult flies. For different larval genotypes, 8-10 replicates of 30 individuals were measured at each ethanol concentration and temperature (~2000 larvae/genotype/temperature). For different adult genotypes, 4-8 replicates of 25-30 flies were measured at each ethanol concentration and temperature (~1000 adults/ sex/genotype/temperature). Asterisks denote genotypes which have significantly different effects on mortality and non-overlapping 95% CI for their associated LD50. The estimate LD50 and standard error are shown and were calculated from logistic regression (logit) analysis of the survival data with genotype as a factor for larvae or genotype and sex as a factor for adults.

assayed larval and adult survivorship after chronic heat stress (48 hours at 32º) or acute heat treatment (30 minutes at 40º) in the presence or absence of an intermediate ethanol concentration (Figure 3.3). Contrary to predictions, T192 does not increase mortality in any of the treatments (Figure 3.3). Heat stress reduces survivorship in larvae ($p<0.001$; Appendix 2, Table A2.3A), and chronic heat-stress reduces ethanol tolerance in adults ($p = 0.005$; Appendix 2, Table A2.3B); under no conditions tested, however, is survivorship higher in K192 than T192 flies. Taken together, these results are inconsistent with the predictions of the hypothesis that balancing selection maintains the Fast and Slow alleles because amino acid T192 increases activity and ethanol resistance at low temperatures but reduces it at high temperatures. Our results indicate that ethanol is toxic, and toxicity increases at high temperature, but T192 either increases ethanol tolerance or has no effect at all temperatures tested.

Another possibility is that other nucleotide polymorphisms, such as those at regulatory sites, increase the fitness of the Fast allele at low temperature and that of the Slow allele at high temperature. We tested this possibility by engineering transgenic flies containing the entire naturally occurring "Fast" haplotype, including the *Adh* coding sequence, introns, and approximately 2 kilobases of 5' and 3' flanking sequences; this construct includes all previously characterized *cis*-regulatory Fast/Slow variants that affect *Adh* expression [106,147]. When larvae were raised at 22º or 27º or exposed to chronic or acute heat shock, the ethanol tolerance of organisms carrying the entire Fast allele was not statistically different from that of the T192-only genotype (Figure 3.2A,vB, Figure 3.3A). In adults, however, the Fast genotype significantly increases ethanol tolerance compared to K192 or T192 alone at both 22º and 27º (Figure 3.2B, D) and when flies are subjected to chronic or acute heat shock (Figure 3.3B). Thus, amino acid polymorphism T192 increases ethanol tolerance in larvae but not adults, whereas linked nucleotide polymorphisms in the Fast allele, presumably at regulatory sites, increase ethanol tolerance in adults but not in larvae. In neither case is there a temperature-dependent tradeoff.

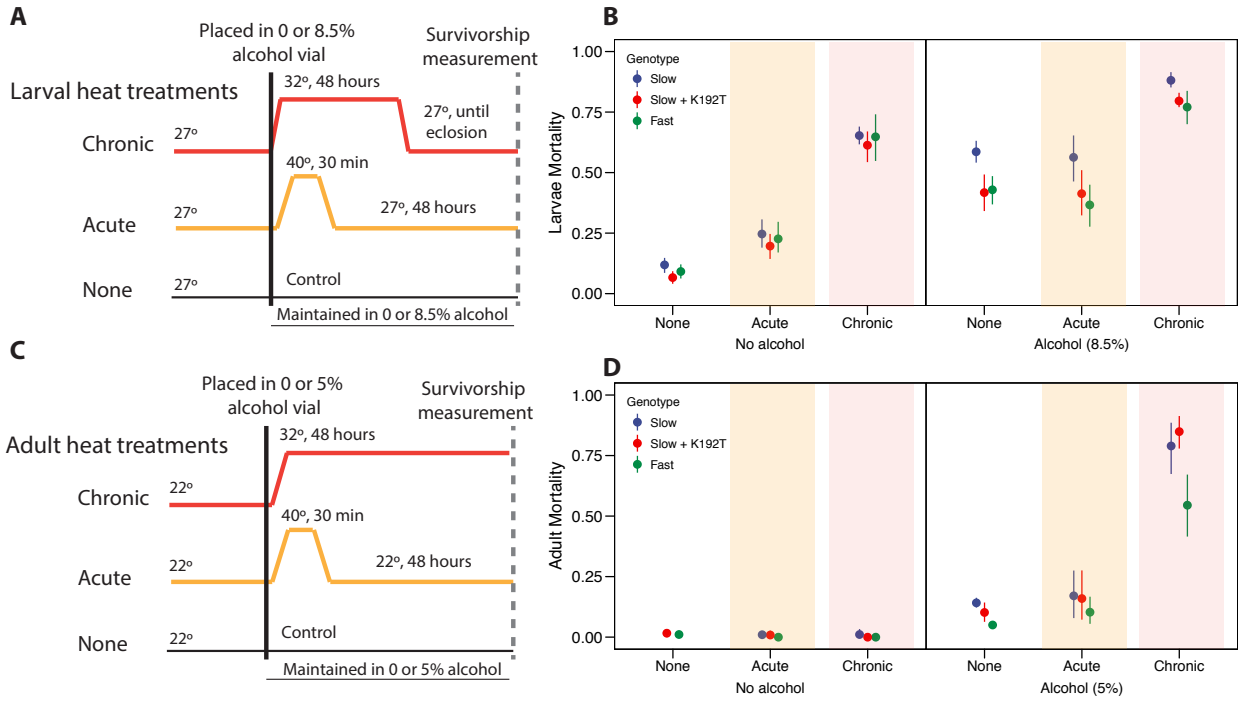

Figure 3.3: The effect of heat-stress on survivorship in presence or absence of alcohol. The T192 amino acid, alone or in the Fast haplotype, is not deleterious when subject to heat-treatments in presence or absence of alcohol. Transgenic larvae and adults were subject to different heat treatments (A, C) in presence or absence of alcohol and measured for their survivorship. Points and error bars show the mean and 95% CI of mortality of 8-10 replicates of pools of 30 individuals (larvae) or between 25-30 individuals (adults). For larvae, both acute and chronic heat stress increased mortality ($p < 0.01$). For adults, chronic heat-stress ($p < 0.01$) increased mortality. Effects of genotype, ethanol treatment, and heat-treatment on survivorship were estimated using logistic regression.

3.3.3 No sequence evidence for the K192T having evolved under long-term balancing selection

Long-term maintenance of the Fast allele due to balancing selection was also hypothesized based on elevated nucleotide diversity around the K192T polymorphism [73]. Those analyses were based on relatively small samples from several geographically distinct populations, raising the possibility that sampling artifacts or demographic differences might counterfeit or dilute a signature of balancing selection [154]. We therefore tested for a signature of balancing selection at site 192 using *Adh* sequences from 163 individuals from a single panmictic population in Raleigh, NC, where both Fast and Slow alleles segregate at intermediate frequencies

[155] and which is located within a previously described latitudinal cline [107,125]. When haplotypes were classified based on the presence of K or T at site 192, nucleotide diversity between classes ($\pi_{between}$) was higher than diversity within classes (π_{within}) in the 250-bp sequence window centered on site 192, as described previously (Figure 3.4A). However, this kind of pattern is always expected around whatever site is used as the focal site to define allele classes. The hypothesis of balancing selection predicts that the excess of between-class diversity ($\pi_{between}/\pi_{within}$) will be greater around the site under balancing selection than around other polymorphic sites with similar frequencies when classes are defined using those sites. We therefore calculated $\pi_{between}/\pi_{within}$ when haplotypes were grouped into allele classes using as a focal site every other intermediate-frequency SNPs (minor allele frequency >0.2) across a 12 kb region surrounding the *Adh* open reading frame, extending well beyond the known regulatory region (Fig. 3.4B). This represents the "background" distribution of $\pi_{between}/\pi_{within}$ produced by the classification process as well as other evolutionary factors that have affected nucleotide diversity in this region. We found that 82% (82/106 intermediate frequency SNPs in the region) of polymorphisms are associated with higher $\pi_{between}/\pi_{within}$ than K192T, providing no evidence of balancing selection at this site (Figure 3.4B, 3.4C). Further, no other sites within the window containing the *Adh* coding sequence and known regulatory elements are associated with an elevated $\pi_{between/within,}$ as would be expected if balancing selection were acting on other specific sites related to *Adh* function.

Taken together, our observations refute all tested predictions of the hypothesis that the K192T polymorphism has been maintained by balancing selection acting on a tradeoff between the enzyme's activity and its thermal stability, which in turn cause temperature-dependent differences in fitness components. The polymorphism does not cause the predicted tradeoff *in vitro*, does not cause the predicted differences in ethanol tolerance in transgenic larvae or adults, and is not associated with a strong sequence signature of balancing selection. Rather, T192 increases the enzyme's rate of ethanol turnover in vitro and increases larval organismal ethanol tolerance; regulatory sites linked to T192 further increase ethanol tolerance in adults. All of these effects are independent of temperature.

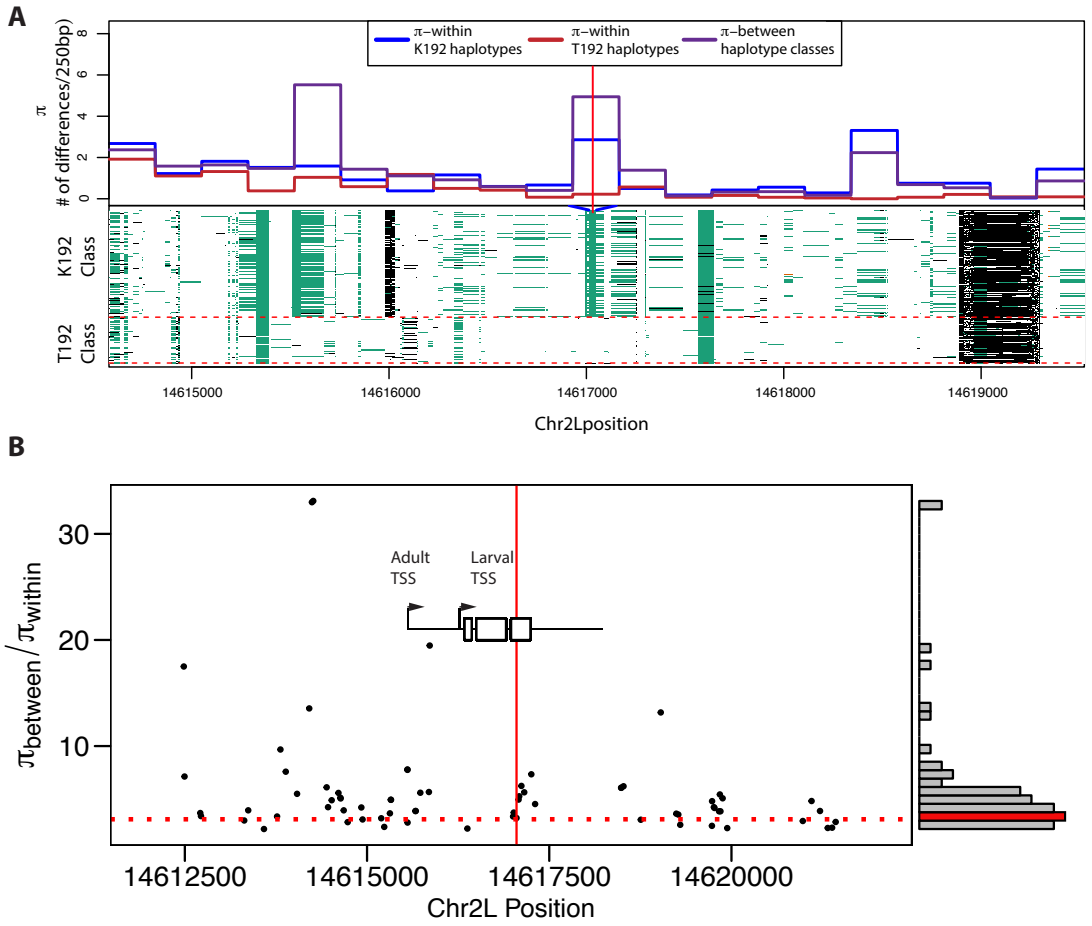

Figure 3.4: Sequence diversity at the Adh locus.
There is no excess of differentiation between K192 and T192 haplotypes as expected under long-term balancing selection. The ratio of diversity between and within allelic classes defined by the K192T polymorphism is similar to the ratio of diversity between and within allelic classes defined by other intermediate frequency polymorphisms. A) The top panel shows average nucleotide diversity in comparisons between two randomly haplotypes with the K192 (Blue), T192 (Red), or between haplotypes that have K192 and T192 (Purple). Bottom panel shows haplotypes (rows) of the K192 or T192 classes. Vertical red line shows position of K192T for top and botto panels. B) Distribution of nucleotide diversity between/ within ($\pi_{between}$/ $\pi_{between}$) haplotype classes when classes are defined by intermediate frequency polymorphisms in the region. Exons of the Adh gene are shown as boxes and the black line denotes regions containing all-previously characterized cis-regulatory variants known to be important for expression. Vertical solid red line shows position of the K192T polymorphism; dotted red line shows the $\pi_{between}$/ $\pi_{between}$ in the window centered on the K192T polymorphism. A histogram of the $\pi_{between}$/ $\pi_{between}$ value is shown on the margins; the bin containing K192T is colored red.

3.4 Discussion

The *D. melanogaster Adh* is an iconic system in evolutionary genetics that has been called the most compelling case of selection acting on genetic variation [18]. Historically, several technical factors made *Adh* an attractive system with which to probe the evolutionary factors that shape natural patterns of genetic variation. The ADH protein is abundantly expressed, and it exhibits substantial variation that can be readily observed using protein electrophoresis [66]. Its enzyme activity could be assayed *in vitro* using a simple spectrophotometric assay, raising the promise that genetic variation in the protein might be experimentally linked to differences in its biochemical function. And ADH's role in ethanol metabolism, together with the physiological and ecological importance of ethanol tolerance in *D. melanogaster,* meant that biochemistry could be plausibly linked to both physiology and fitness. Adh therefore became one of the first systems in which an integrated experimental account connecting genotype, molecular function, phenotype, and fitness could be attempted [1,18].

One of the first questions investigated was why variants of ADH are maintained and also segregate along latitudinal clines in several regions [107,122]. Several observations and experimental results led to the formulation of a specific hypothesis that connected numerous biological levels. Substantial circumstantial evidence was amassed to support this hypothesis, but technical limitations prevented decisive tests. *D. melanogaster* strains containing the Fast and Slow haplotypes were assessed for ethanol tolerance [115,120,127,156], and the biochemical activity of cell lysates were studied for enzyme activity [120–123,128,129], yielding results consistent with the balance/tradeoff hypothesis. But these strains (and their lysates) differed not only at the key amino acid but at many linked sites at or near *Adh* -- including those that affect *Adh* expression -- and also at many other loci that might affect ethanol tolerance or temperature sensitivity, such as components of the heat shock response [99,147–149]. Efforts to breed strains better controlled for genetic background [75,106] or to biochemically separate the fraction of lysate containing alcohol dehydrogenase activity [86] made the experiments somewhat more precise, but even these could not fully isolate the amino acid polymorphism from other genetic differences. Numerous

other studies produced conflicting results concerning different aspects of the effects of the Fast haplotype on various phenotypic parameters [86,98,139–141,143–146], possibly because each one incorporated different forms of this background genetic variation.

Today, methods of genetic manipulation make it possible to express and purify heterologous ADH proteins *in vitro* and to engineer transgenic flies that are identical except at the putatively critical amino acid site. We used these techniques to specifically test the predicted effects of the amino acid polymorphism on activity, thermostability, organismal heat sensitivity, and ethanol tolerance. This key difference in design presumably explains why our work did not corroborate any of the functional predictions of the stability/activity balancing selection hypothesis suggested by previous work. Using heterologously expressed, purified, and concentration-matched ADH proteins and direct biochemical measurements, we found that T192 increases alcohol dehydrogenase activity *in vitro* in a temperature-independent fashion and has no measurable effect on thermal stability relative to K192. Moreover, flies carrying the ADH T192 polymorphism are more ethanol tolerant than otherwise identical flies bearing K192. Introducing the *Adh* regulatory region further increases ethanol tolerance, but there is no evidence of increased heat sensitivity in either strain of flies. Even one of the sequence signatures of balancing selection at the K192T polymorphism is no longer apparent when contemporary sequence samples are used and the background distribution in nucleotide diversity is accounted for.

These results reject the specific hypothesis that balancing selection, mediated by a stability/activity tradeoff, explains the Fast/Slow genetic variation in natural populations. Our work does not rule out the possibility that other kinds of balancing selection acting across latitudinal gradients might maintain *Adh* genetic variation [157]. Indeed, innumerable hypotheses could be articulated--and tested--concerning selective factors that might act on genetic variation in *Adh* via biochemical and physiological properties other than an activity/thermal stability tradeoff. Although we did not detect a sequence signature of balancing selection, large population sizes and high recombination rates in *D. melanogaster* rapidly erode the patterns necessary to detect balancing selection [16,111]. However, genome-wide analyses that have been done for *D. mela-*

nogaster have not identified the *Adh* locus as a target of other selective events [158]. Further, although the Fast allele increases ethanol tolerance with no tradeoff in heat resistance, these experiments were conducted in a specific strain of flies; we cannot rule out the possibility that in certain genomic contexts the Slow allele might confer fitness equal to--or even higher than--the Fast allele.

Our findings also contradict the general idea that there is an intrinsic biophysical tradeoff between enzyme activity and thermal stability [131–135,159]. Indeed, numerous other studies have identified mutations that increase both activity and stability [137,160], or increase stability without reducing activity or function [112,161,162]. Although mutations that increase activity often decrease stability [133,134], this observation may simply reflect the fact that the majority of all mutations reduce stability [138]. Proteins from organisms that inhabit lower-temperature environments are often less stable than those from high-temperature environments [137], but this pattern does not provide evidence for a tradeoff. The same relationship would arise if proteins need only be stable enough to predominantly occupy folded conformations, with excess stability causing no fitness benefit or decrement [136].

Although our results strongly refute the stability/activity balancing selection hypothesis, they suggest an alternate hypothesis that may partially explain the long-term maintenance of the Fast and Slow alleles. The Fast haplotype increases ethanol tolerance, irrespective of temperature, suggesting that it may be selected for in environments with higher levels of ethanol, while Fast and Slow may segregate with nearly equal fitness in lower-ethanol environments. If ethanol is more abundant in the environments of *D. melanogaster* at higher latitudes and some gene flow occurs across this gradient, this scenario could also explain the clinal distribution of the alleles. This hypothesis is consistent with the observation that flies from higher latitudes have greater ethanol tolerance [98,126] and the fact that that nucleotide diversity is very low in the Fast haplotype class [73], as expected if the haplotype originated recently and swept to increased frequency in some populations [16,102]. Our experiments provide evidence for the links between genotype, function, and fitness components in the presence of ethanol predicted by this hypothesis.

But we know of no current evidence to support the crucial premise that ethanol in *D. melanogaster*'s environment is directly related to latitude. Further, it is possible that the clinal distribution of the Fast/Slow polymorphism could arise not from selection but from the peculiar demographic history of *D. melanogaster [163]*. Future experiments are therefore necessary to corroborate, refute or modify the hypothesis we propose to explain the observed patterns of genetic variation in *Adh* and its associated phenotypes.

3.5 Methods

Protein expression and purification: Protein coding sequences of the ADH K192 (Slow) enzyme variant was synthesized by *de novo* synthesis (GenScript), and the T192 (Fast) variant was generated using the Phusion Site-Directed Mutagenesis method (ThermoFisher). The K192 and T192 coding sequences were cloned into pLIC-maltose binding protein (MBP) plasmids to generate fusion proteins with an N-terminal hexahistidine tag. Plasmids were verified by sanger sequencing and transformed into E. coli BL21 (DE3) Rosetta Cells.

ADH enzymes were expressed by first growing transformed cells overnight in 5 mL of LB at 37*C and subsequently diluting them into 500 mL LB flasks the next morning. Cells were grown at 37*C with shaking until they reached an OD_{600} = ~ 0.6, at which point protein expression was induced using 0.5 mM isopropyl B-D thiogalactoside (IPTG). Cells were harvested by centrifugation after cultures reached an OD_{600} between 1.5 – 1.8 and frozen.

To purify enzymes, frozen cell pellets were lysed using Bper, Lysozyme, and DNAseI. Lysate was then passed over a nickel-affinity HIS-trap chromatography column to isolate the (His_6) MBP-ADH protein using standard methods. The (His_6)MBP tag was subsequently removed by treating the fusion protein sample with tobacco etch virus (TEV) protease overnight and then using HIStrap and Cation columns to separate and purify the ADH enzymes. Purified ADH samples were flash frozen in 10% glycerol solution and stored at -80*C until they were used in experiments.

Stability measurements: Stability of the K192 and T192 variants was measured using circular dichroism to monitor how secondary structure was lost across a temperature ramp. CD signal was monitored at 222 nM across a temperature range of 22 – 60 degrees in a 0.1m cuvette for protein samples at a concentration of 10uM in 50 mM Sodium Phosphate buffer using a Jasco J-1500 CD Spectrometer, using a 2C/min temperature ramp. Samples were equilibrated at each temperature for 2 minutes before measurements were taken and two iterations were taken for each measurement temperature. Three independent replicates were done for each enzyme variant.

Proportion of folded protein was estimated using a two-state denaturation model, and a 5-parameter logistic regression curve was fit to the data to estimate the temperature at which 50% of the molecules had denatured.

Enzyme activity assays: The effect of temperature on reaction rates was measured by characterizing the activity of 500 nM of K192 and T192 enzymes under saturating co-factor and substrate conditions at different temperatures.. Reaction mixtures containing 1mM nicotinamide adenine dinucloetide (NAD), 50 mM sodium phosphate (pH 7.6), and ethanol or isopropanol at 200 mM concentration were incubated at 22, 30, or 37 degrees for one hour. The reaction was then started by adding 25 ul of 4uM enzyme that had been kept at room temperature, and fifteen replicates were done at each temperature. The rate of reaction was measured by monitoring absorbance at 340 nm, which provides an estimate of the concentration NADH, a byproduct of ethanol oxidation. Observations from the first 90 seconds of adding the enzyme were used to estimate velocity. The effect of temperature on activation energy and statistical significance for a temperature by genotype effect of the reaction was estimated by fitting the arrhenius equation to observed reaction rates per unit enzyme (k_{cat}) as a function of temperature and genotype using the lm() function in R.

The effect of heat-incubation on retention of catalytic activity was measured by incubating the two variants at different temperatures (22º – 50º) for one hour and then adding 25 ul of the incubated enzyme to reaction mixtures kept at 22º. Reaction rates were estimated as described above. Between ten and fifteen replicates were done for enzymes at each incubation temperature.

Transgenic organisms: *D. melanogaster* flies carrying Adh alleles that differ only at their Adh gene were made using the PhiC31-attp transgenesis system, as described in detail in previous work [109,164]. Injection constructs were constructed with three different haplotypes--a naturally occurring K/Slow haplotype derived from a Canton-S strain, the K/Slow haplotype + K192T substitution, and a naturally occurring T/Fast haplotype from a Florida strain (FL9-20)—and injected into an Adh null strain. The injected strain contains the attP landing site ZH-86Fb and phiC31 integrase (genotype: y[1]M{vas-int.Dm}ZH-2A w[*]; Adh[fn6]cn[1];M{3xP3-RFP.attP}

ZH-86Fb). Injected flies were backcrossed to the pf86 strains and F1 transformants were identified by their eye color because they had a w^+ allele. These transformants were subsequently crossed to sibs to yield transformant strains that were homozygous for the transformed allele. Lines were tested for correct insertions and sequence via PCR.

Ethanol survivorship Assays: Adult ethanol tolerance was measured by placing 25-30 adults aged between 2-4 days into vials with Whatman paper soaked with 600ul of 3% sucrose solution and a variable concentration (0 – 10%). For each genotype, between 4-8 replicates were done at each ethanol concentration for each sex. The proportion of surviving flies after 48 hours was measured at each concentration and the relationship between survivorship and ethanol concentration, genotype, and sex was assessed with logit regression with the quassibinomial link function, as implemented in the glm() function in R. The magnitude and statistical significance of these effects and of LD_{50} were estimated from the fitted model.

Larval ethanol tolerance was measured by taking replicates of 30 larvae at the second-third instar transition and placing them on drosophila media containing different concentrations of ethanol (0-15%) and measuring survivorship to adulthood. The Genesee Scientific Molasses Formulation media was used and made according to instructions but with a ratio of 140g powder/L of water. To reduce alcohol, alcohol was stirred into the media after the food had cooled to below 60ºC and vials were immediately plugged. For each genotype between 8-10 replicates of larvae were measured at each concentration. The effect of ethanol concentration and genotype on survivorship and ethanol LD50 was estimated as described for adults.

Heat stress assays: Replicate populations of ~30 adults or larvae for each genotype--reared at 27ºC and 22ºC, respectively—were placed in vials with normal media or media supplemented with an intermediate concentration of ethanol and then subjected to either chronic or acute heat-shock. Organisms treated with a chronic heat-shock were placed for 48 hours in a 32ºC incubator. For adults, proportion of surviving were flies were immediately measured. For larvae, the

vials were returned to their rearing at temperature and proportion of flies surviving to adulthood was measured. Organisms treated with an acute heat-shock were placed for 30 minutes in a 40ºC incubator and then returned to their rearing temperature. For adults and larvae, the number of flies surviving after 48 hours and to adulthood were measured, respectively. The effect and statistical significance of ethanol, genotype, heat-stress, and interaction terms was estimated using logit regression in R. The interaction terms included were those that were found to significantly increase in model fit, as deemed by the F-test in the anova( ) function in R.

Sequence analysis: Sequence analysis was done using the DGRP dataset, with sequence data extracted per instructions given in the DPGN pipelines. Individuals who had missing information at >20% of sites in the Adh region were removed. Average pairwise nucleotide diversity between and within haplotypes carrying different Adh alleles (K192/T192) or other intermediate frequency SNPs (freq = 0.2 – 0.5) was measured using custom scripts written in R.

Chapter 4: Evolution of protein specificity: Insights from ancestral protein reconstruction

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4.1 Summary

Specific interactions between proteins and their molecular partners drive most biological processes, so understanding how these interactions evolve is an important question for biochemists and evolutionary biologists alike. It is often thought that ancestral proteins were systematically more "promiscuous" than modern proteins, and specificity often does evolve by partitioning and refining the activities of multifunctional ancestors after gene duplication. However, recent studies using ancestral protein reconstruction (APR) have found that ligand-specific functions in some modern protein families evolved *de novo* from ancestors that did not already have those functions. Further, the new specific interactions evolved by simple mechanisms, with just a few mutations changing classically recognized biochemical determinants of specificity, such as steric and electrostatic complementarity. Acquiring new specific interactions during evolution therefore appears to be neither difficult nor rare. Rather, it is likely that proteins continually gain and new lose activities over evolutionary time as mutations cause subtle but consequential changes in the shape and electrostatics of interaction interfaces. Only a lucky few of these, however, are incorporated into the biological processes that contribute to fitness before they are lost to the ravages of further mutation.

4.2 Introduction

Specific molecular interactions – between enzymes and substrates, receptors and ligands,

and transcription factors and DNA response elements – underlie most cellular processes. How these interactions evolve has long been a source of interest for biochemists and evolutionary biologists [165–169]. A widely accepted hypothesis is that ancestral proteins were generalists, recognizing a broad set of ligands, from which modern highly specialized enzymes then evolved by gene duplication, partitioning and refining ancestral activities among the descendants [166–168,170–174]. This view holds that the genetic paths to new functions are long, making them unlikely to evolve after a gene duplication but before deleterious mutations eliminate the extra copy [172,173,175]. If all the activities of highly specific enzymes were already present in their ancestors, however, evolution could simply partition those functions [176] or refine them under selection after gene duplication [174,177]. (For definitions of specificity and promiscuity, see Table 4.1.)

This idea is appealing for several reasons. First, some present-day enzymes carry out secondary, biologically relevant "moonlighting" activities [178–180], so it is plausible that ancestral proteins might also have had such activities, which subsequently evolved into the primary functions of extant proteins. Second, it has been thought that evolving new biochemical activities should be genetically difficult, requiring many mutations in the context of tight constraints imposed by protein architecture, whereas losing activities already present in an ancestral protein would be much easier. For example, comparative studies of enzyme specificity have shown that homologous enzymes with distinct substrate specificities often differ by many residues, and efforts to transform the specificity of one protein into that of another by swapping a small number of amino acids at structurally important sites often fail [181]. Moreover, directed evolution studies found that new substrate-specificities could evolve more easily and along shorter genetic paths by optimizing low-level side activities present in the starting protein than by acquiring activity on an entirely new substrate [182–184]. This work also showed that some secondary activities could evolve without significantly compromising a protein's primary function [182,185], providing a plausible scenario by which ancestral proteins could have slowly acquired the functions that were ultimately partitioned among their descendants.

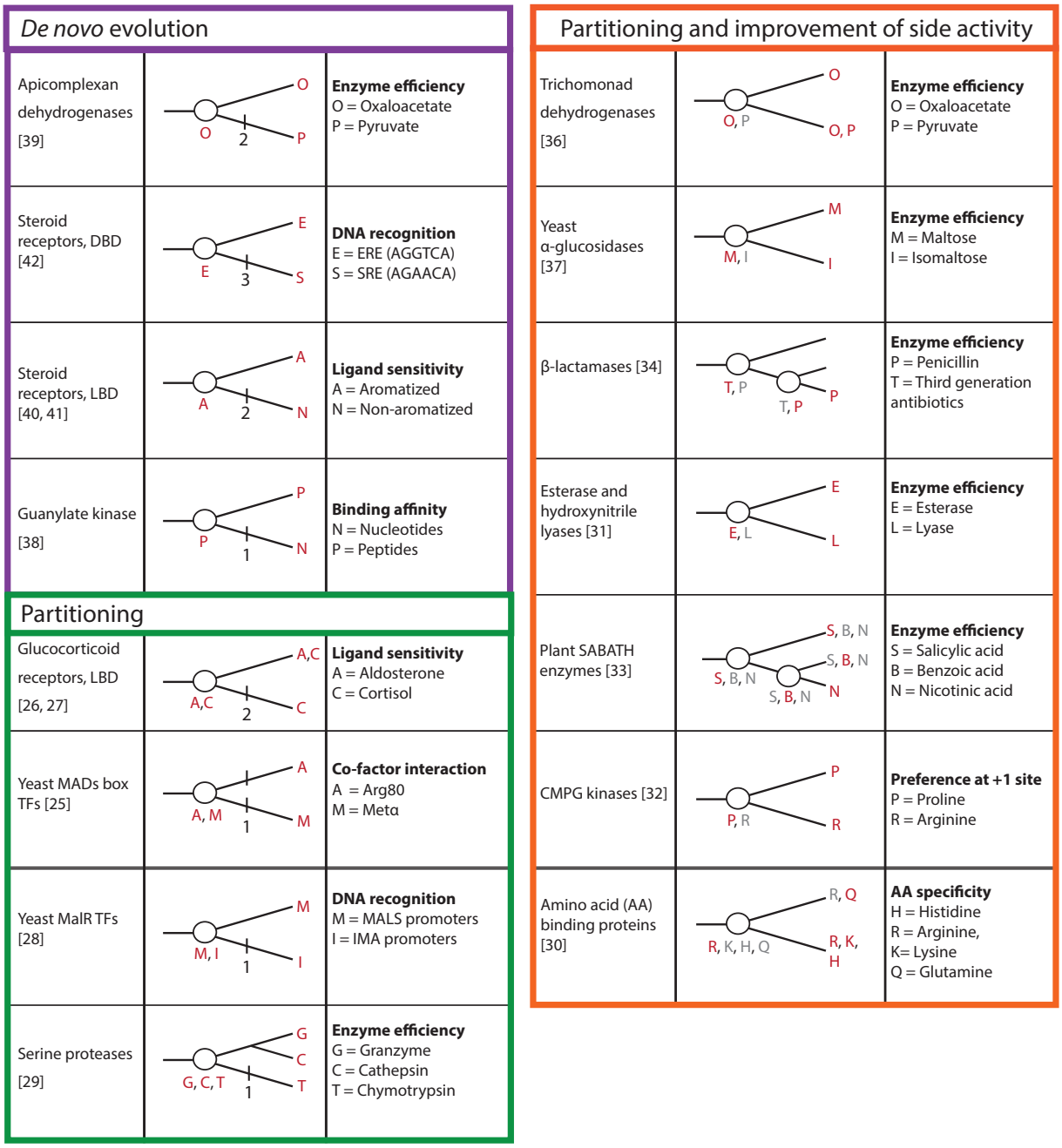

Table 4.1: Ancestral reconstruction studies of specific molecular interactions.
For each protein family studied, the cladogram shows the specific biological or biochemical property assayed, its distribution among extant and reconstructed ancestral proteins (circles). Protein families are grouped on the basis of their evolutionary history: *de novo* evolution of a new specific function after gene duplication (purple), partitioning of functions from a multifunctional ancestor (green), or partitioning of functions from a multifunctional ancestor with refinement of an ancestral activity in one or both lineages (orange). For studies that dissect genetic mechanisms, the number of large-effect replacements necessary to recapitulate the shift in specificity is shown on the lineage on which they occurred. Red letters, major function or activity (high affinity, catalytic efficiency, etc.); gray, minor function (lower affinity, etc.).

These observations are consistent with the idea that specific proteins can readily evolve from generalist ancestors, but they are not sufficient to establish that historical evolution always -- or even usually -- occurred this way, or that it is genetically difficult for proteins to evolve specificity *de novo*. First, the existence of moonlighting activities in many present-day proteins does not necessarily imply that all the functions of extant proteins are derived from secondary functions in their ancestors. Second, directed evolution regimes typically exert very strong selection pressures to produce defined *in vitro* chemical activities very rapidly, with high mutation rates, very strong selection, few pleiotropic constraints, and little or no opportunity for drift; they are therefore unrepresentative of the long-term historical processes of protein evolution [22]. Finally, comparative studies of homologous proteins are limited in their capacity to identify the minimal causes of functional differences, because long periods of sequence divergence and epistatic interactions among substitutions might obscure relatively simple mechanisms by which proteins in the deep past diverged in function [186,187].

4.3 Reconstructing evolution of molecular specificity using ancestral protein reconstruction

Recent developments in ancestral protein reconstruction (APR) make it possible to directly address the historical evolution of specificity. This strategy begins with statistical inference of ancestral protein sequences, followed by gene synthesis, expression of ancestral proteins, and characterization of their physical and functional properties [188]. These techniques allow hypotheses about the specificity or promiscuity of ancestral proteins to be experimentally assessed. Further, amino acid changes from key intervals of phylogenetic history can be re-introduced into reconstructed ancestral proteins, so hypotheses about the mechanisms by which changes in specificity evolved can be directly tested.

Recent studies have used APR to reconstruct the evolution of specificity (Table 4.1). Of these, many have found that the specificities of related extant proteins were partitioned [189–193] or enhanced [194–201] from a common ancestor with multiple functions. These

studies establish that multifunctional ancestors sometimes give rise to specific descendants, but they do not reveal the genetic or biochemical mechanisms for how those functions evolved in the first place. Several recent studies, however, have documented protein families in which the specific biological functions of present-day proteins evolved *de novo* from ancestral proteins that lacked those functions, and they reveal the mechanisms by which those new functions evolved [202–206]. Here we survey these findings, discussing three case studies in which different kinds of biological specificity evolved *de novo*: substrate-specificity of an enzyme, ligand-specificity of an allosterically regulated protein, and DNA-specificity of a transcription factor.

4.3.1 Substrate-specificity of metabolic enzymes

Members of evolutionarily related enzyme families typically share similar catalytic chemistry but have diversified in substrate specificity [169,207,208]. APR can clarify how this diversity evolved by measuring the activity of ancestral proteins against the substrates of their descendants and then identifying the historical causes, both genetic and structural, that can recapitulate the evolution of the derived specificity.

A recent study examined how highly distinct substrate specificities evolved in a pair of related enzymes – the malate and lactate dehydrogenases (MDH and LDH) of apicomplexan alveolates, a phylum of single-celled eukaryotes [202]. These two enzymes, which play key metabolic roles, are related by a gene duplication event that occurred 700-900 million years ago. MDH catalyzes reduction of oxaloacetate, whereas LDH catalyzes reduction of pyruvate. Each enzyme is highly specific, with virtually no activity against the substrate of its paralog. The two ligands differ in that oxaloacetate is longer with a C3 hydroxyl group and a charged carboxylate at C4, whereas pyruvate is smaller and ends with a hydrophobic C3 methyl.

The authors reconstructed and characterized the common ancestral protein from which the Apicomplexan MDHs and LDHs arose (AncM/L) by duplication (Figure 4.1a). They found that AncM/L was not multifunctional; rather, like extant MDHs, it was highly specific for oxaloacetate, with virtually no activity on pyruvate and a preference for oxaloacetate (ratio of

k_{cat}/K_m for the two substrates) > 10^7. In contrast, the subsequent common ancestor of all extant LDHs (AncL) was, like extant LDHs, specific for pyruvate, preferring it over oxaloacetate by >200,000-fold. A discrete switch in specificity – not partitioning of activity from a generalist ancestral protein – therefore occurred during the phylogenetic interval between AncM/L and AncL.

To identify the best candidates among the 65 substitutions that took place in this interval, Boucher et al. determined the X-ray crystal structures of the two ancestral proteins. Two of the changes were in the active site loop – a six amino acid insertion and an arginine to lysine substitution at a nearby site (Arg102Lys). When introduced together into AncM/L, they recapitulated evolution of the derived specificity, causing a 60,000-fold increase in catalytic efficiency (k_{cat}/K_m) on pyruvate and a >40,000-fold reduction on oxaloacetate, thus shifting preference by more than ten orders of magnitude. The loop insertion alone conferred the new function almost entirely, increasing pyruvate efficiency by a factor of >12,000, while only weakly affecting oxaloacetate activity (Figure 4.1B). Introducing just Arg102Lys moderately reduced catalysis of oxaloacetate activity without affecting pyruvate activity, yielding a weaker enzyme on either substrate. Whichever mutation occurred first, the resulting intermediate form existed only transiently, because AncL and all known descendants possess both sequence features and are pyruvate-specific.

The ancestral crystal structures provided a physical explanation for how the loop insertion conferred the novel activity on pyruvate (Figure 4.1C). AncM/L was specific for oxaloacetate because the positively charged side chain of Arg102, which paired with oxaloacetate's negatively charged carboxylate group, was left unsatisfied by pyruvate's methyl. The insertion placed a Trp residue near this position, which improved hydrophobic packing of the methyl and moved residue 102 out of the active site into a solvent-exposed position, allowing that residue's electrostatic potential to be satisfied when pyruvate is bound. The structures also showed that the loop and substitution together would likely reduce activity on oxaloacetate because they would leave that substrate's carboxylate unpaired in the active site.

Thus, LDH appears to have acquired its pyruvate reductase function *de novo* by a single

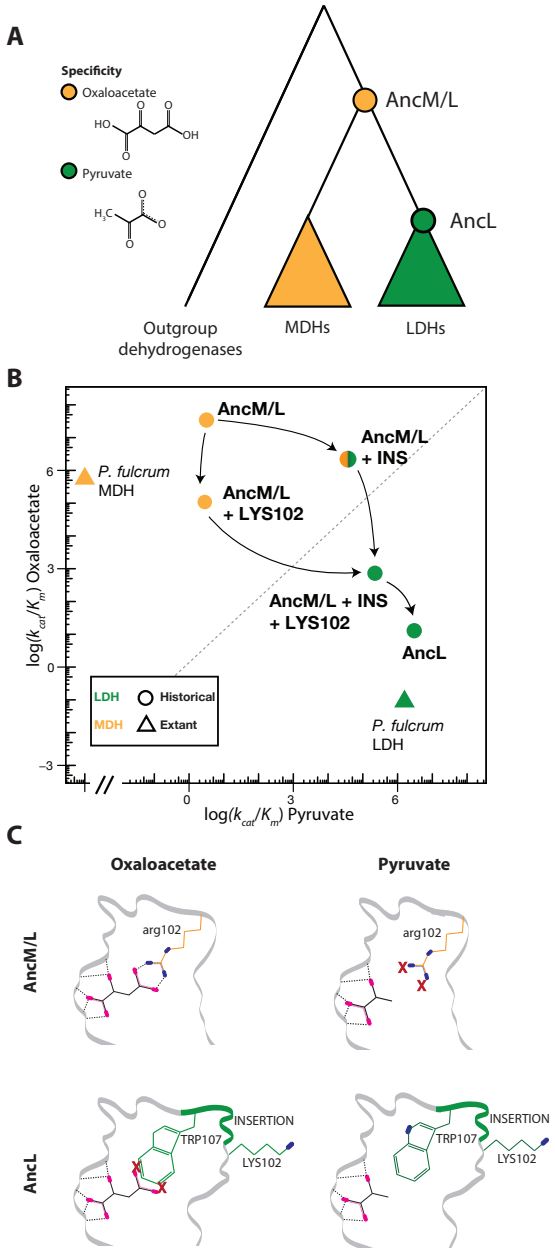

Figure 4.1:
Evolution of lactate and malate dehydrogenase specificity in Apicomplexa [40].
A) Evolutionary relationships of paralogous dehydrogenases and reconstructed ancestral proteins (circles), colored by their substrate-specificity for oxaloacetate (orange) or pyruvate (green). B) Catalytic efficiency of extant and reconstructed dehydrogenases on oxaloacetate and pyruvate. Effect on ancestral activity of the historical amino acid replacement Arg102Lys (LYS102) and an insertion in the active site loop are shown; arrows show possible evolutionary paths from oxaloacetate- to pyruvate-specificity. C) Mechanism for the evolution of pyruvate specificity in LDH. The top row shows a portion of the structure of the ancestor AncM/L with oxaloacetate (left) and pyruvate (right); the bottom row shows the derived ancestor AncL. Sites affected by the key genetic changes are shown, with colors corresponding to panel A. Oxygen atoms, magenta; nitrogen, blue. Dashed lines show hydrogen bonds from enzyme to substrate; X, unsatisfied electrostatic potential.

genetic change that altered a key electrostatic contact between protein and substrate; a second change then abolished the ancestral function by further altering electrostatic contacts. APR was essential to this discovery: extant MDH and LDHs share only ~50% sequence identity, and swapping these two active site changes between paralogs is no longer sufficient to interconvert their functions [202]. This observation indicates that subsequent epistatic substitutions modified the effect that the key sequence changes had when they occurred historically.

4.3.2 Ligand-specificity of allosteric recognition

Many proteins bind and are allosterically regulated by specific interactions with other proteins or small molecules. Our laboratory has studied the evolution of this phenomenon in the vertebrate steroid receptors (SRs), a family of allosterically regulated transcription factors with diverse specificity for steroid hormones [190,193,204,209,210]. The receptor's ligand binding domain (LBD) binds a steroid molecule in its hydrophobic core, which causes the transcriptionally active conformation to be favored. There are two phylogenetic classes of SRs, which also correspond to the chemistry of their ligands: the estrogen receptors (ERs) bind steroids with an aromatized A-ring and a 3-hydroxyl, and the non-aromatized steroid receptors (naSRs), which bind androgens (AR), progestogens (PR), glucocorticoids (GR), and mineralocortioids (MR), most of which have a 3-carbonyl (Figure 4.2a). Eick et al. [203] reconstructed the LBD of AncSR1, the common ancestor of all present-day vertebrate SRs, which duplicated to produce the two classes (Figure 4.2a). This protein was found to be highly specific for estrogens, activating transcription in response to low doses of vertebrate estrogens but causing no activation in the presence of a large panel of nonaromatic steroids. In contrast, the subsequent common ancestor of all the naSRs (AncSR2) displayed the opposite specificity, with a very sensitive response to a variety of nonaromatized steroids and no activation by any estrogens. A discrete shift in functional specificity therefore occurred on the branch between these two ancestral proteins, with functional sensitivity to a new ligand evolving *de novo* and the ancestral ligand-sensitivity being abolished.

Extant ERs and naSRs differ at about 70 percent of sequence sites, and even AncSR1 and AncSR2 differ by 171 substitutions, suggesting that the mechanism for the difference in specificity might be very complex. But APR, along with an X-ray crystal structure of the ancestral protein, allowed the authors to identify two substitutions that occurred during the interval between AncSR1 and AncSR2 that can account for most of the evolutionary change in specificity [210]. Reversing these two sites in AncSR2 to their ancestral states conferred a >100,000-fold shift in preference for estrogens vs. non-aromatized steroids. Introducing the derived states into AncSR1

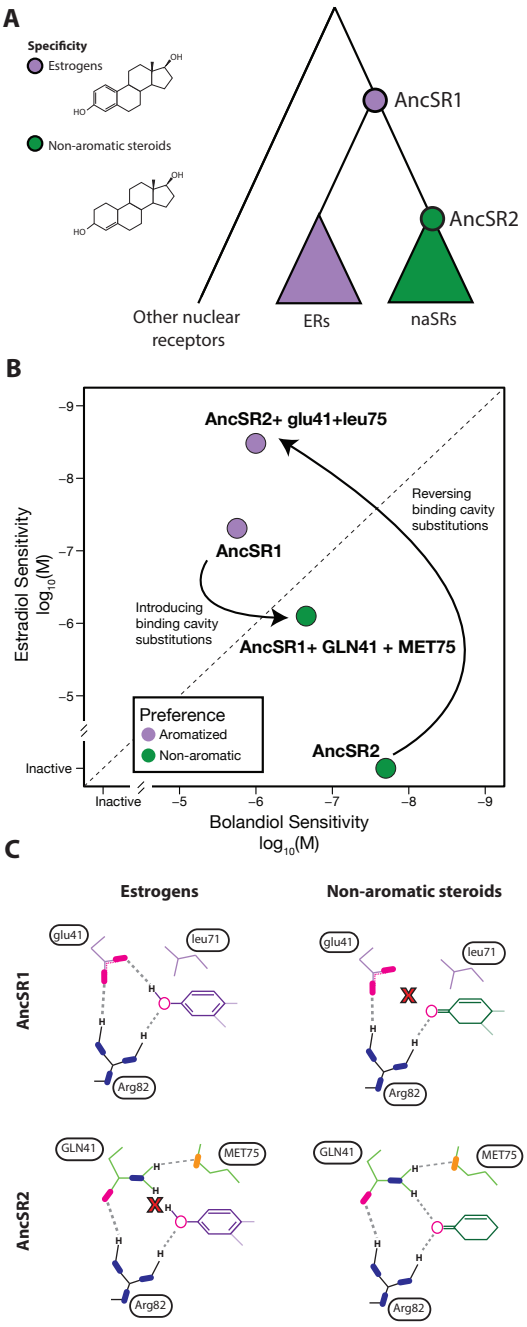

Figure 4.2: Evolution of ligand specificity in vertebrate steroid receptors [41, 46]. A) Evolutionary relationship of estrogen receptors (ERs) and non-aromatized steroid receptors (naSRs). Circles represent reconstructed ancestral proteins (AncSR1 and AncSR2), colored by their ligand specificity for estrogens (purple) or steroids with a nonaromatized A-ring (green). B) Effect of two historical substitutions on preference for aromatized or non-aromatized steroids. Each protein is plotted according to the ligand concentration at which half-maximal activation of a luciferase reporter was achieved (EC50), for the estrogen estradiol and the otherwise identical but nonaromatized androgen bolandiol. Effect of ancestral and derived states (lower and upper case, respectively) of historical mutations glu41GLN and leu75MET are shown when introduced into the reconstructed ancestral proteins. C) Mechanism for large-effect historical mutations. Top row shows AncSR1 with ancestral states in complex with the aromatized ring of estrogens (left) and nonaromatized steroids (right). Bottom row shows AncSR2 with derived states. Key residues are labeled; Arg82 is conserved among all steroid receptors. Oxygen atoms, magenta; nitrogen, blue; sulfur, orange. Dashed lines show hydrogen bonds from ligand to receptor; X, unsatisfied electrostatic potential.

also conferred activation by nonaromatized steroids and dramatically shifted the receptor's preference from estrogens to these hormones (Figure 4.2b).

The two key mutations altered the electrostatics of the pocket and its complementarity to polar groups on the two ligands (Figure 4.2c). AncSR1 coordinated the hormone's A-ring using a network of hydrogen-bond acceptors that are fully satisfied with estrogens, including a Glu that

accepts a hydrogen bond from the 3-hydroxyl of estrogens. This network effectively excluded nonaromatized steroids, most of which contain a 3-carbonyl, because that complex would contain an excess of unsatisfied hydrogen-bond acceptors in the pocket; even nonaromatized steroids that contain a potential donor at this position present it at the wrong angle to pair with the Glu [209]. The two key substitutions replaced this Glu with Gln and introduced a second polar residue into the pocket, which created a new arrangement of donors and acceptors that could pair with nonaromatized steroids but left the 3-hydroxyl of estrogens unpaired. Molecular dynamics and hydrogen-deuterium exchange experiments both showed that the derived protein's hydrogen bond network is consistently satisfied in the active conformation when nonaromatized steroids are bound. But with estrogen in the pocket, AncSR2 explores numerous suboptimal conformations in which the hydrogen bonding potential of the pocket is unfulfilled, unless water molecules are drawn into the protein's interior, disrupting the active conformation. Thus, as in MDH/LDH evolution, just two historical mutations are sufficient to in the ancestral background to remodel the binding site, changing specific electrostatic complementarity to the ancestral ligand into specific complementarity to the derived ligand.

4.3.3 DNA specificity of transcription factors

Regulation of cell state and activity depends on specific interactions between transcription factors and DNA response elements (REs) in the vicinity of target genes. Evolution of DNA specificity has been studied using APR in several families of TFs, including the DNA-binding domain of SRs. All SRs bind as dimers to palindromes of a 6 base-pair "half-site." ERs bind preferentially to EREs -- palindromes of AGGTCA – whereas naSRs bind to SREs (palindromes of AGAACA and variants) [211]; ERs activate reporter transcription effectively from EREs but not SREs, and the converse is true of naSRs (Figure 4.3a).

Reconstruction and characterization of the DBDs of AncSR1 and AncSR2 illuminated the evolutionary trajectory and mechanisms by which these specificities diversified [206]. AncSR1's DBD, like its LBD, was highly specific, activating reporter expression from EREs but showing

no activation on SREs. AncSR2's specificity was inverted, activating from SREs but not at all on EREs. Thus, SRE-mediated transcriptional activity evolved *de novo* from an ancestor that did not already possess that function. Follow-up biochemical studies showed that this change in biological specificity was mediated by massive changes in affinity between AncSR1 and AncSR2 – an increase in affinity for SRE by a factor of ~200 and a ~400-fold reduction in ERE affinity, together yielding an 80,000-fold shift in relative affinity. Underscoring the point that low-level chemical activities are not necessarily relevant in biological terms, AncSR1 did have weak affinity for SREs, and AncSR2 had weak affinity for EREs, but in both cases those affinities were too low to mediate transcriptional activation.

The shift in specificity was caused in large part by a few genetic changes. The authors focused on three key substitutions that occurred during the AncSR1-AncSR2 interval, were conserved in different states between the two classes of SR, and are located on the proteins "recognition helix" (RH), which inserts into the DNA major groove and contacts the variable base pairs in the half-site. Experiments showed that these were indeed large-effect historical substitutions: reversing them in AncSR2 to the ancestral state was sufficient to switch the protein's DNA specificity in reporter assays from SRE back to ERE and to confer a ~1,500-fold increase in relative affinity for EREs (Figure 4.3b). Conversely, when the derived states were introduced into AncSR1, they increased specificity for SREs by four to five orders of magnitude by both increasing SRE affinity and reducing ERE affinity. When introduced together with a group of permissive mutations that nonspecifically affected DNA affinity, these three substitutions yielded a DBD that mediated robust transcription on SRE.

In this case, too, the mutations reversed the protein's specificity by changing electrostatic complementarity to the ligands. X-ray crystal structures and MD simulations of the ancestral proteins showed that the three substitutions did not establish any new positive interactions to SREs; rather, they affected exclusionary interactions, removing steric clashes and unsatisfied polar/charged groups that had reduced the AncSR1's affinity for SRE, while introducing new incompatibilities with ERE. For example, AncSR1 bound SRE poorly because an ancestral

glutamate on AncSR1's recognition helix sterically clashed with two methyl groups on the DNA that are unique to SRE, leaving the glutamate and several other charged or polar moieties in the interface unsatisfied. In AncSR2, this glutamate is replaced with a glycine, which both removes the charged side chain and its unsatistfied potential and relieves the steric clash, increasing SRE affinity without introducing any new positive interactions. In turn, the derived side chains also left several polar groups on the ERE unpaired. Thus, specificity was shifted not by generating new positive interactions with the derived RE but by changing the negative determinants of specificity to exclude the ancestral binding site and allow binding to the derived site.

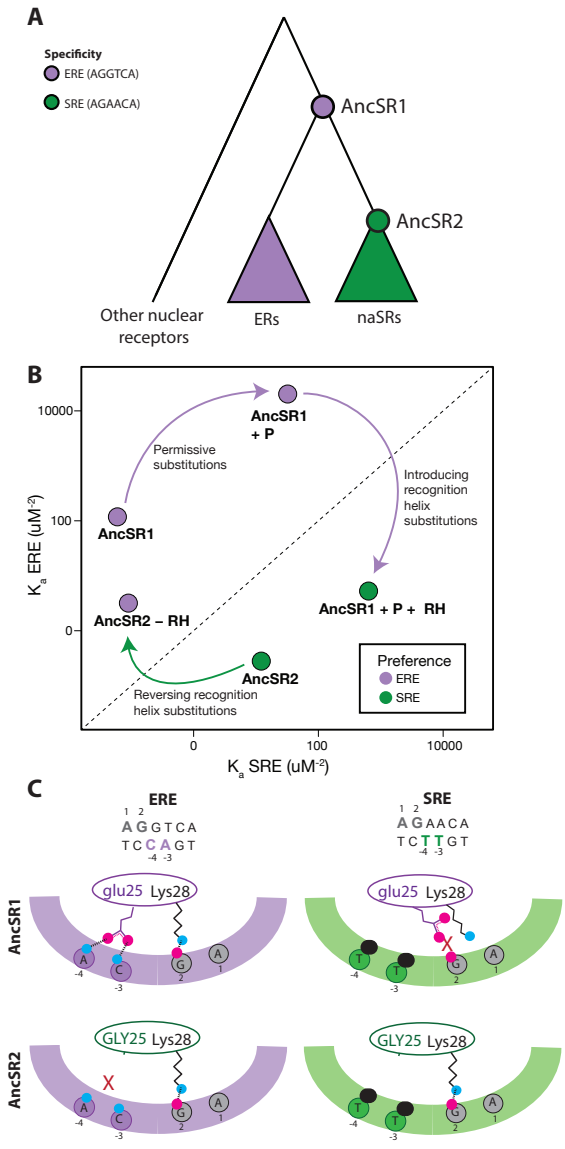

Figure 4.3: Evolution of DNA specificity in vertebrate steroid receptors [43].
A) Evolutionary relationship of DNA binding domains (DBDs) of ERs and naSRs. Circles represent the ancestral proteins, AncSR1 and AncSR2. Colors show preference for estrogen or steroid response elements (ERE, purple; SRE, green). B) Effect of historical amino acid replacements on DNA affinity for ERE and SRE, shown as the association constant (Ka) for each motif in a fluorescence anisotropy binding assay. Purple arrow shows the preference-switching effect of introducing the derived states at three residues in the recognition helix (+RH) and the non-specific effect of permissive changes (+P) on AncSR1. Green arrows show the effect of reverting to the ancestral residues at the RH sites (-RH). C) Mechanism for the effect of historical RH substitution glu25GLY on specificity. The top and bottom rows shows AncSR1 and AncSR2, respectively, in complex with ERE (left) and SRE (right). The protein's RH is shown as an oval, and the DNA major groove as a colored arc; bases that participate in specific interactions are shown, numbered according to their strand. Bases that differ between the REs are colored. Side chains at site 25 and the conserved residue Lys28 are shown. Magenta, oxygen atoms; blue, nitrogen; black, methyl group. Hydrogen bonds are shown with dashed lines. X, unsatisfied hydrogen bonding potential. A steric clash between methyl groups on the SRE and the ancestral glu25 repositions that side chain, disrupting two ancestral hydrogen bonds; substitution of GLY25 relieves this clash and allows SRE binding.

4.4 Evolution and the biochemical mechanisms of specificity

4.4.1 Why can specificity evolve through few genetic changes?

These studies used APR to investigate different kinds of molecular specificity – between enzymes and substrates, receptors and ligands, and transcription factors and DNA – but they reveal similarities in the genetic and physical mechanisms of functional evolution. Although the number of cases is relatively small (Table 4.1), three major lessons emerge. First, the specific biological functions of extant proteins have in some cases emerged *de novo* following gene duplications; although such *de novo* trajectories are not the most frequent kind of history observed, they can and do occur. Second, when new specific interactions with molecular partners evolved, they did so through a small number of large-effect substitutions, with other historical sequences changes exerting permissive, fine-tuning, or inconsequential effects [162,212,213]. Third, the mechanisms by which these large-effect substitutions conferred new functions were relatively simple, involving steric clashes and changes in polar interactions between protein and ligand.

These evolutionary observations are consistent with fundamental knowledge concerning the biochemical causes of protein specificity span [214,215]. Although a satisfied hydrogen bond makes only a small contribution to affinity in solvent -- because the system forms the same number of hydrogen bonds whether or not the complex is bound -- an unsatisfied polar or charged group on a hydrophobic interface can strongly reduce affinity by causing the bound complex to make fewer total hydrogen bonds than the unbound state [214,215]. A polar or charged group can therefore discriminate strongly against ligands that provide no complementary group to fulfill the residue's electrostatic potential. Steric clashes can also have dramatic effects on specificity, because Pauli repulsion effects are associated with very high energies; a protein structure may adjust to minimize such clashes, but these changes often disrupt other interactions in the complex.

These findings imply that specificity should be evolvable via one or a very few mutations. A substitution that introduces a donor or acceptor onto a hydrophobic interface can increase the

free energy of binding by up to 5 kcal/mol, decreasing affinity by 5,000-fold [215]. Conversely, a substitution that removes an existing polar or charged group – or satisfies an existing unpaired group – will increase affinity by the same amount. A single substitution that accomplishes both of these changes, leaving a donor or acceptor unsatisfied with one ligand and satisfying a previously unpaired group – can therefore shift specificity by up to 10^7-fold. Substitutions that induce ligand-specific steric clashes or introduce multiple unsatisfied donors or acceptors into a binding site can have even larger effects. These are precisely the kinds of mechanisms – and magnitude of effects – that APR studies show drove *de novo* acquisition of specific functions during historical protein evolution.

4.4.2 Why are trajectories from generalist ancestors so common?

If specific functions have evolved *de novo* in some cases by such simple mechanisms, then why does there appear to be a preponderance of protein families in which specific paralogs evolved by partitioning from a multifunctional ancestor (Table 4.1)? There are three requirements for paralogs with distinct specific functions to evolve. Without specifying an order in which they must occur, those requirements are: 1) a gene duplication must take place; 2) the distinct biochemical activities found in the extant paralogs must be differentially gained and/or lost among the paralogs in a way that produces distinct specificities; and 3) the genes and their relevant functions must be preserved to the present. We propose that the third requirement often represents the rate-limiting factor in long-term protein evolution and is the primary cause of differences in the observed frequency of trajectories in which specific paralogs evolve from multifunctional ancestors vs. those in which new specificity evolves *de novo*.

Both *de novo* and partitioning histories require a gene duplication, so the first requirement *per se* cannot account for the preponderance of trajectories in the former category. What about the second requirement: is it difficult or unlikely for biochemical activities to originate *de novo?* The answer is almost certainly no. Virtually all extant proteins have a large number of chemical side-activities – sometimes high-efficiency ones -- that do not contribute to the organism's func-

tion or fitness (reviewed in [207,216]). Further, as the studies we have discussed make clear, new specific activities can often evolve via just one or a few substitutions. It is therefore likely that proteins are continually gaining and losing secondary biochemical activities during evolution because of the stochastic processes of mutation and drift. The purported difficulty of evolving new biochemical activities is thus not a plausible explanation for the preponderance of histories in which the ancestor already had the functions of its descendants. Indeed, new activities must have originated during evolution at some point to have been present in the ancestor and/or its descendants; partitioning histories merely push these events further back in time.

We propose that the key difference between the two kinds of trajectories is how they affect the third requirement – that duplicated genes and biochemical activities be retained over evolutionary time. Unless genes are conserved by purifying selection, mutation and drift erode their sequences quickly, leading to loss of function, expression, or the reading frame itself. To be preserved over long periods of evolutionary time, then, a duplicate gene and its biochemical activity must contribute to fitness. In some cases, such as during adaptation to strong selection pressures like novel antibiotics or pesticides, a new chemical activity might be immediately advantageous and become selectively maintained [217–221]. But in most cases, the divergent specificities of proteins involve endogenous ligands, and these activities are more slowly integrated into the organism's biology – its development, physiology, or metabolism, for example. This suggests that subsequent genetic changes at other loci, affecting such processes as production of a substrate or ligand, utilization of the product, or regulation of the gene's expression, were likely required to incorporate the new activity into the organism's biological processes and fitness. Because additional genetic changes across multiple genes are involved, this process is likely to be a major limiting factor in the long-term evolution of most new protein functions.

A key difference between *de novo* and partitioning evolutionary histories is that the latter leave more time for a new biochemical activity to become biologically significant and subject to purifying selection. In *de novo* evolution, a biochemical activity must originate after gene duplication and then be incorporated into the biological functions of the organism before the activity

or the gene is lost. In contrast, partitioning from a multifunctional ancestor requires the new activity to evolve before the duplication, and it can become biologically significant either during this pre-duplication period, when the single copy of the gene is protected from degenerative mutation by purifying selection, or after. Partitioning histories therefore leave more time than *de novo* histories do for this rate-limiting step to occur and are for that reason—not because evolving a new function is genetically difficult—more likely to produce pairs of paralogs with distinct specificities. A partitioning trajectory also requires the ancestral activities to be partially lost in a complementary fashion among the paralogs, but this step is unlikely to be rate-limiting because it can occur by degenerative mutation and neutral drift [222].

A corollary of this argument is that there is no reason to believe that ancient proteins were in general more multifunctional or promiscuous than extant ones. When we say that an extant protein evolved some specific function *de novo*, this means only that the ancestral protein from which it evolved did not have that function, not that it did not have any – or many -- other side activities. Today's proteins will eventually be the ancestors of tomorrow's. Between now and then, many promiscuous activities will be gained and lost; only those that both become biological functions constrained by natural selection and undergo gene duplication will ultimately produce paralogs with distinct specificities. Of the innumerable biochemical activities that extant proteins now possess or will acquire, the only ones that scientists of the future will observe – and the only ones for which we will demand an account of their ancestors – are these happy few.

Chapter 5: Conclusion

The study of biological adaptations—or as Darwin called them "organs of extreme perfection and complication"--has always occupied a special place in studies of life and natural history [223]. There is a strong case to be made that Darwin's most original contribution in the development of evolutionary biology was not the idea of the common origin of life, but the articulation of a framework where the remarkable features of biological life could be viewed as results of a process with causes and effects. This framework made the study of natural variations tractable and nature's most inscrutable mysteries into amenable to study and investigation with the methodologies of science. One thing we have learned in the history of studying adaptations, however, is that they are among the more complicated problems to study, requiring continuous rearticulation of the scientific question and discovery of methodologies that can appropriately address them. Nature, it appears, does not easily reveal the secrets of her most wondrous creations.

Major limitations of the work presented in this thesis, and of studies of adaptation in general, stem from the complexity of the subject matter. Studying adaptation in a scientific way first requires identifying an adaptation, and therein begins one of the major difficulties: traits that seem as if they must have evolved adaptively are often "spandrels," the non-adaptive byproducts of other processes [3]. We rarely know if the forms and functions we view as adaptations are actually adaptations. An additional complication is that many types of plausibly adaptive phenomena—such as visual or auditory signals—are imperceptible to unaided human observation and such features remain absent from our inquiries altogether. Focusing on genotype instead of phenotype does not solve the problem: false signatures of selection abound and even the strongest signatures of selection may be caused by selfish genetic elements that are maladaptive [2]. In addition, as seen in cases concerning evolution of protein specificity, remarkable functional transitions can happen as a result of very few genetic changes but such cases will go undetected in most tests of selection as a byproduct of the diagnostic methods. The question—how did this trait evolve?—requires first addressing the difficult question of "is this trait adaptive?" Second,

the traits that exist today are products of a stochastic and contingent evolutionary history , about which we have relatively sparse information. Even when a compelling explanation can be provided for an adaptive phenotype, the role of chance events--genetic and environmental--in determining the evolution of that adaptation will often remain, making it impossible to determine to what degree the adaptive feature that evolved was the best, the luckiest, or the only one possible. Methodologies have emerged in the last decade that have yielded some insight into role of historical contingency in specific cases of functional evolution but the application of these methods, particularly for studying contingency at the level of organismal phenotypes, remains restricted to relatively few study systems. The prevalence of stochasticity and contingency in biological evolution makes it difficult to establish which principles are generalizable and to what extent. All of these factors make adaptation not only a difficult field to study, but one that is fraught with incorrect inferences.

The results discussed in this thesis highlight these challenges. In testing two long-standing hypotheses, this work demonstrates that neither are supported by the data; in one case, there is no evidence for adaptive evolution and the other case suggests possible adaptive evolution through different selective causes than originally speculated. The capacity to detoxify alcohol is almost certainly a derived capacity of the *D. melanogaster* species that allows it to survive and thrive in habitats that are lethal to other organisms. Yet, the evolution of the ADH enzyme had little to do with the evolution of this trait in *D. melanogaster,* despite the intuitive connection. However, this work suggests that when a second bout of putative alcohol adaptation occurred that further differentiated populations of *D. melanogaster,* structural and regulatory mutations in *Adh* likely did play a role but did so without the predicted temperature-associated cost. In both cases, refuting the adaptive hypotheses has resulted in a better—albeit more complicated—understanding of how adaptation to an environmental resource happened evolutionarily. This raises new questions: If ADH mutations can drive differences in ethanol tolerance between populations, why might such mutations not have driven divergence between species? Were changes in ADH expression adaptive, or did *D. melanogaster* adapt via other, hereto unexplored, physiologi-

cal routes that bypassed the ethanol catabolism pathway? If the Fast allele confers a benefit in presence of ethanol and temperature does not preclude its existence at hotter temperatures, then what—if anything—does? Further, at the level of natural history, why have flies from colder environments repeatedly evolved higher ethanol tolerance? The value of this work, in one sense, is that it makes clear some of the next questions that should be addressed in future studies of this trait.

The second, and perhaps more important, contribution of the work presented in this thesis is its methodology. Adaptive hypotheses necessarily make predictions about the functional and fitness consequences of historical genetic changes, but such hypotheses have been difficult to test. Combining ancestral sequence reconstruction with engineering of transgenic organisms can make many of these previously untestable hypotheses directly testable, as it did for ADH. How to interpret results obtained from such studies will be a matter of debate, but such debates will hopefully lead to specific conjectures and refutations that ultimately enrich our understanding of natural processes. Lewontin wrote, regarding the study of evolutionary biology in the genomic era, that "The greatest methodological challenge that it now faces is to connect the observations about the outcome of evolutionary processes to the tradition of experimental functional biology." [67] It is my hope that this work, however incrementally, has contributed to confronting this challenge.

Appendix 1

Supplementary figures and tables for Chapter 2: Experimental test and refutation of a classic case of molecular adaptation in *Drosophila melanogaster*

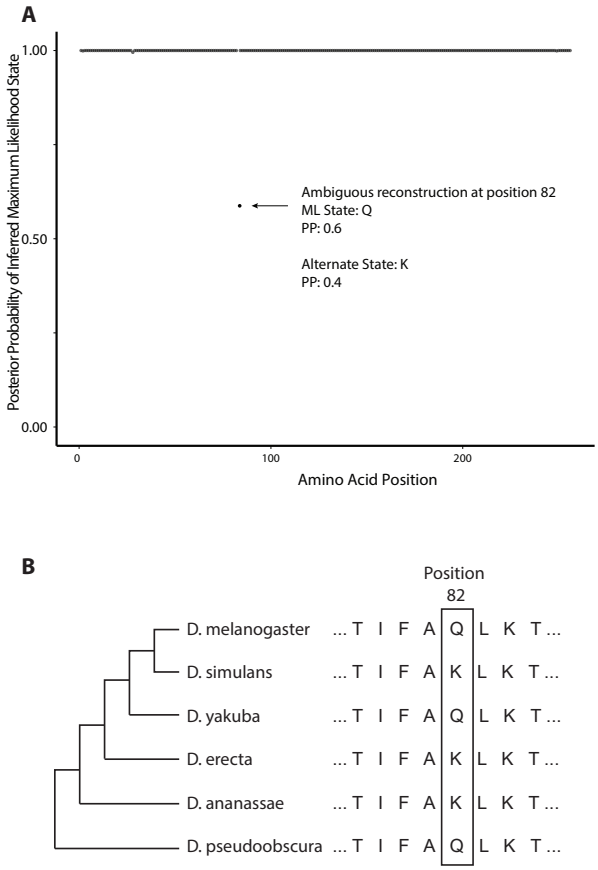

Figure A1.1: Reconstruction of AncMS. The sequence of AncMS is reconstructed with extremely high statistical confidence. **(A)** Posterior probability (PP) of the maximum PP site across sequence sites. Only one site (arrow) is ambiguously reconstructed in AncMS. (**B**) States observed in extant sequences at ambiguously reconstructed site 82.

Figure A1.2: Alcohol dehydrogenase activity with different alcohol. ADH activity using isopropanol as a substrate did not change between ancestral and *D. melanogaster* alleles. Reaction rates measured at different isopropanol concentrations were fit to a Michaelis-Menten model. **A)** Plot of initial reaction velocity across isopropanol concentrations for purified ADH proteins of different genotypes. Each point shows the mean and 95% confidence interval of six reactions at each dose. **B&C)** Estimated maximal catalytic turnover rates (k_{cat}) and Michaelis-Menten constant (K_m), each with 95% confidence interval inferred by nonlinear regression from the data in panel A. Activity for the same enzymes using ethanol as a substrate (data from Figure 2), are shown for comparison.

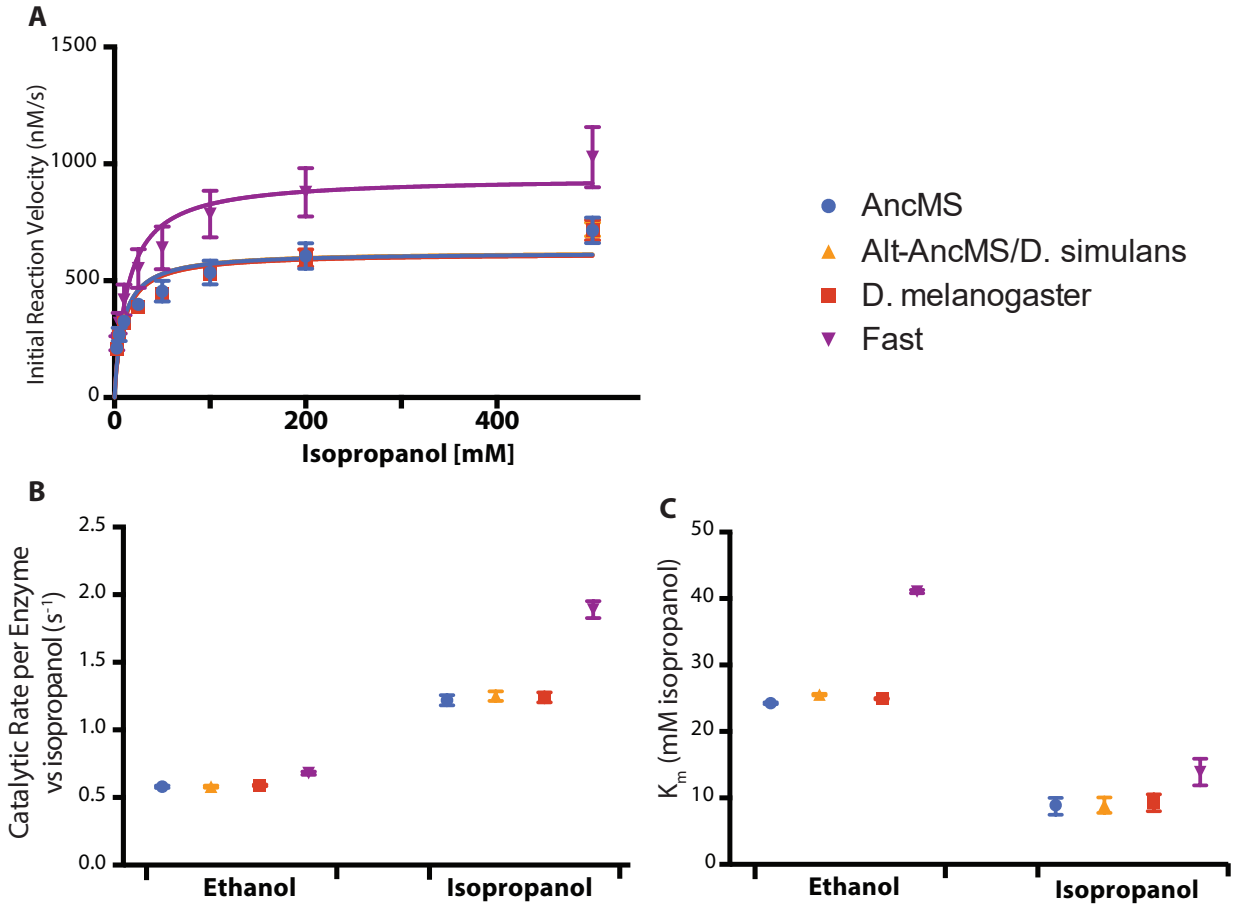

Figure A1.3: Within and between species variation in the ADH coding sequence. Divergence and polymorphism data used for the McDonald-Kreitman tests. States at each variable sequence site (numbered by nucleotide) are shown. Nonsynonymous substitutions and polymorphisms are highlighted in red.

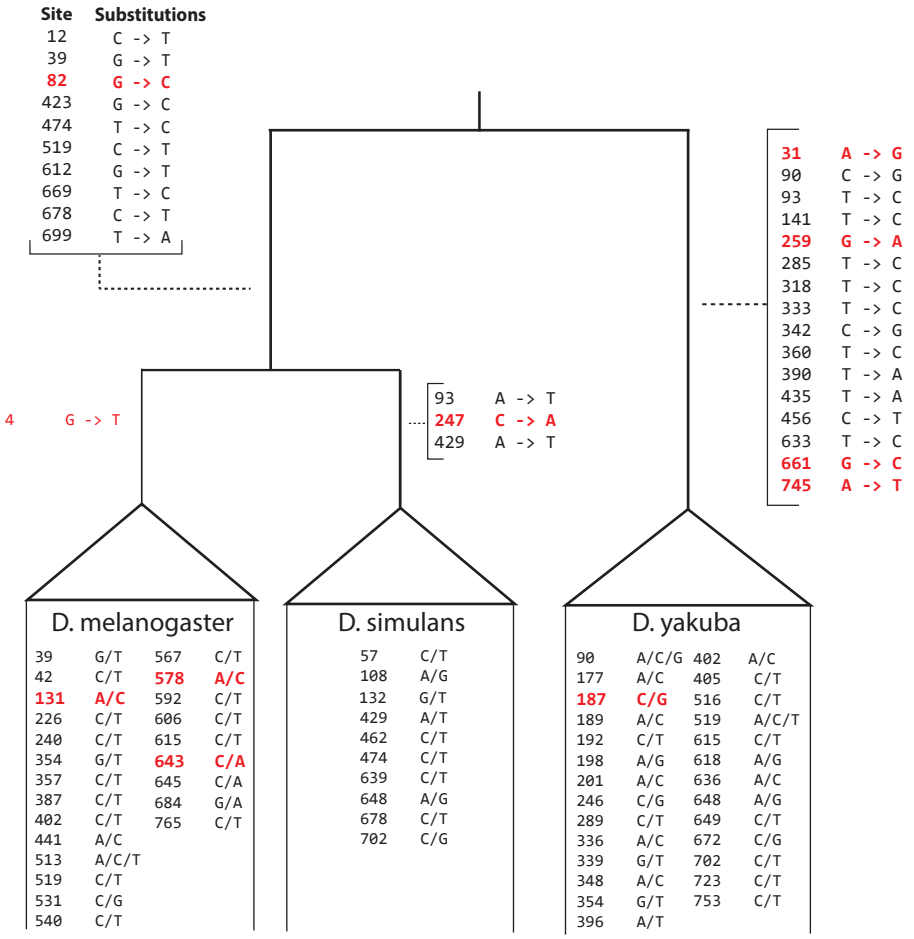

Figure A1.4: ADH activity in lysate from independently transformed transgenic genotypes. Replicate transgenic strains within each genotype do not statistically differ from each other in ADH activity of whole-fly homogenates, and only the Fast allele is distinct from the ancestral *D. melanogaster* allele. **A)** The maximum reaction rate, normalized by total protein in the homogenate, for adults from different transgenic strains of each ADH construct is shown. For each strain, the mean and 95% confidence interval for 15 homogenates is shown. P-values are from unpaired t-tests. **B)** Comparisons of maximum reaction rate for transgenic animals of different ADH coding sequence assessed by ANOVA. Mean differences in activity between strains with the ancestral *D. melanogaster* ADH and other alleles were calculated from Dunnet's test to account for multiple comparisons. Error bars correspond to 95% CI.

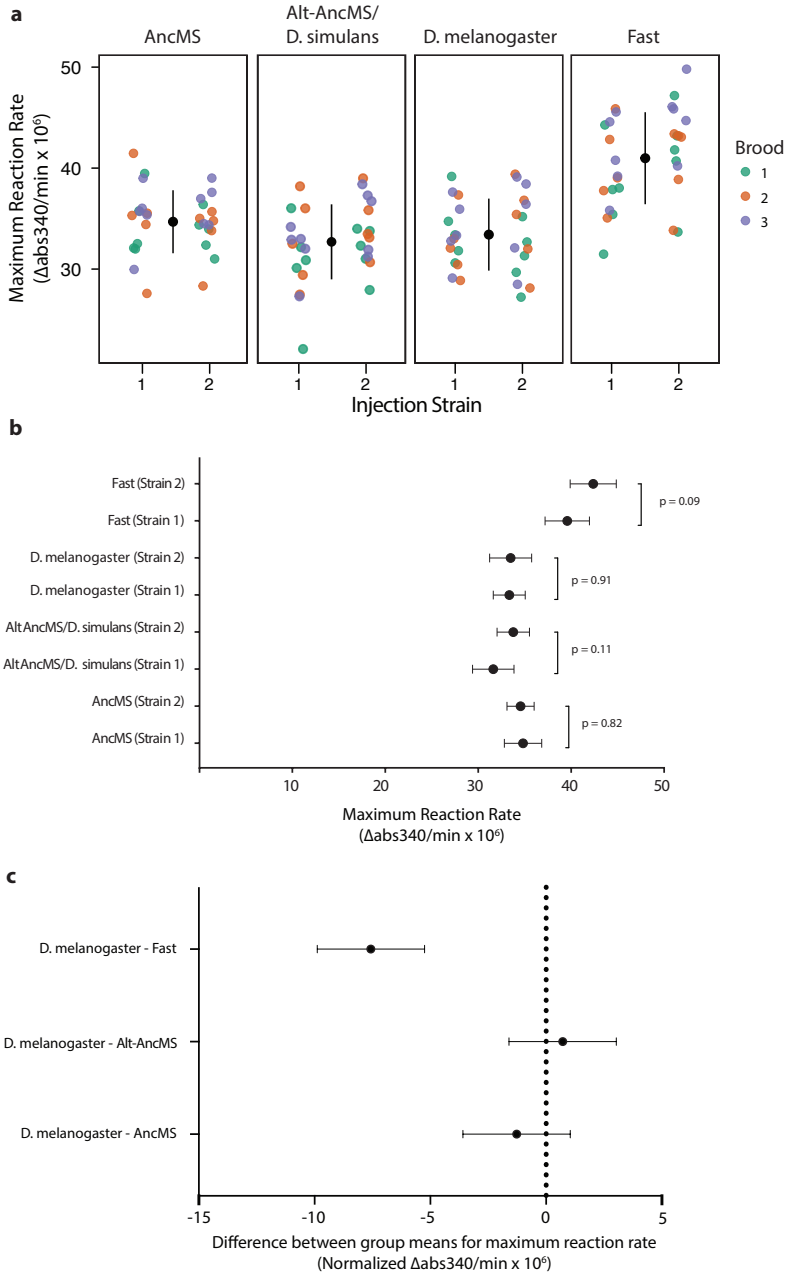

Table A1.1: Kinetic parameters of ADH alleles expressed and purified from bacteria. The best-fit value of each parameter with its 95% confidence interval is shown. Only the Fast allele (bold) differs from the other genotypes.

Extended Table 1

Genotype	Substrate	K_m (95% CI) mM	k_{cat} (95% CI) s^{-1}
AncMS	Ethanol	24.2 (20.7 – 27.7)	0.580 (0.558 – 0.602)
Alt-AncMS/D. simulans	Ethanol	25.5 (22.2 – 28.7)	0.582 (0.563 – 0.602)
D. melanogaster (Slow)	Ethanol	24.9 (22.2 – 27.7)	0.595 (0.577 – 0.612)
Fast	Ethanol	**41.0 (34.3 – 47.8)**	**0.678 (0.645 – 0.710)**
Alt-AncMS/AncMS	Isopropanol	87.4 (63.3 – 111)	1.24 (1.17 – 1.32)
D. simulans	Isopropanol	89.2 (67.4 – 111)	1.24 (1.16 – 1.31)
D. melanogaster (Slow)	Isopropanol	92.7 (68.1 – 117)	1.25 (1.18 – 1.31)
Fast	Isopropanol	**138 (100.2 – 177)**	**1.88 (1.76 – 2.00)**

Table A1.2: McDonald-Kreitman test using different sets of ADH data, including the originally published 1991 alignment[24]; an expanded dataset with more new polymorphism data added; only those substitutions and polymorphisms from each branch leading to each of the three major species analyzed; and alignments from which the sequences of one of the species have been removed. The number of synonymous and nonsynonymous divergences between species (Dn and Ds) and polymorphisms (Pn and Ps) are shown, with the p-value associated with the null hypothesis of neutral evolution in each case.

Extended Table 2

Dataset	Dn/Ds	Pn/Ps	p-value
McDonald-Kreitman (1991)	7/17	2/42	0.007
MK + modern data	7/23	5/59	0.049
D. melanogaster	1/0	3/21	0.16
D. simulans	1/2	0/10	0.231
D. yakuba	4/12	1/28	0.047
D. yakuba + D. simulans	6/23	1/38	0.037
D. yakuba + D. melanogaster	6/21	4/49	0.079
D. melanogaster + D. simulans	2/2	3/31	0.076

Appendix 2

Supplementary figures and tables for Chapter 3: selection via a stability-activity tradeoff deoes not maintain clinal variation in the *alcohol dehydrogenase* enzyme of *D. melanogaster*

Figure A2.1: The X-ray crystal structure of the D. melanogaster ADH. The K192T polymorphism (spheres) does not contact the substrate (orange), co-factor (green), or change the protein secondary structure. Insets show magnification of the active site with positions 191-193 shown as sticks and position 192 displayed as balla nd sticks with either a Lys (left, lightblue backbone) or a modeleted Thr (right, magenta backbone). Atoms for positions are colored by element: Blue--nitrogen, Red--oxygenThe structural position and effect of the K192T polymorphism. B) Circular dichroism spectral profile of K192 (blue) and T192 (red). Dotted lines corresponds to 222nm, the wavelength monitored to calculate the melting curves of the enzyme variants

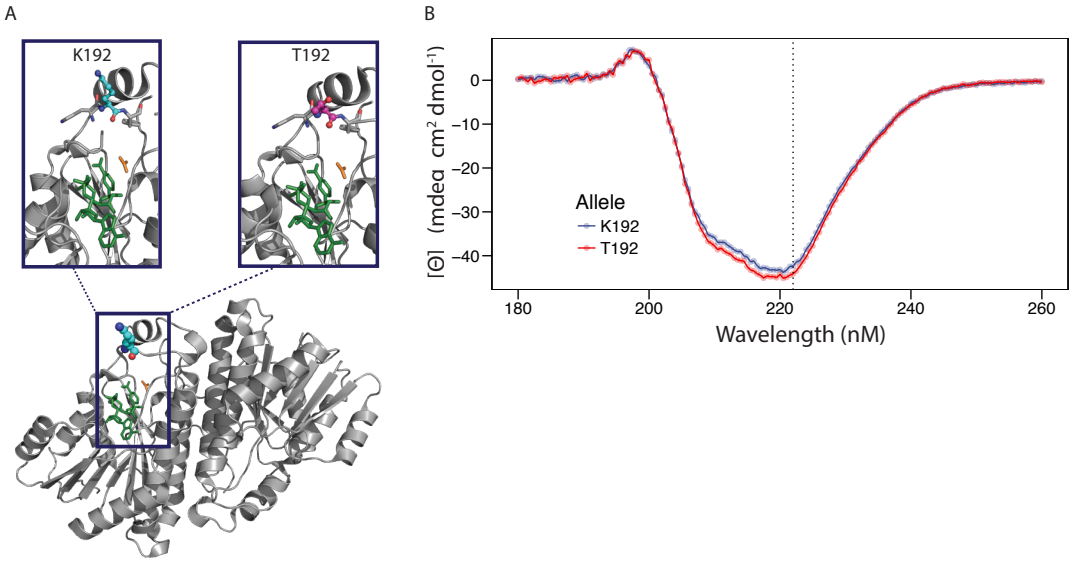

Figure A2.2: The effect of different heat-stress treatments on flies. Flies of genotypes become incapacitated when treated to 40º for longer than 30 minutes (**A**) or at temperatures higher than 32º for over 24 hours (**B**). Dots represent replicate vials containing 25-30 adult flies.

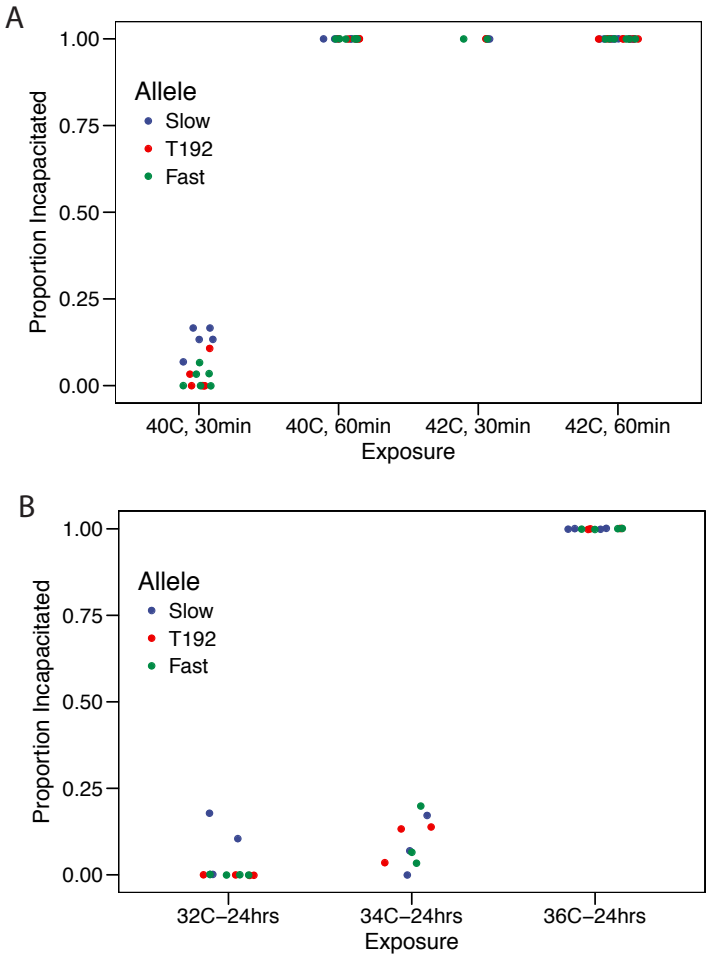

Table A2.1: The effect of ethanol concentration and genotype on larval survivorship. The T192 mutation confers higher ethanol tolerance but there is no temperature at which the T192 mutation is deleterious. Tables A and B show summary statistics from best-fit logit models for larval ethanol survivorship at 22ª and 27º, respectively.

A

term	estimate	std.error	statistic	p.value
(Intercept)	4.4542	0.2766	16.1032	< 0.0001
Concentration	–0.5652	0.0321	–17.6287	< 0.0001
K192T	0.0906	0.1713	0.5290	0.5973
Fast haplotype	0.3352	0.1729	1.9388	0.0539

B

term	estimate	std.error	statistic	p.value
(Intercept)	2.6722	0.1397	19.1246	< 0.0001
Concentration	–0.3883	0.0170	–22.7836	< 0.0001
K192T	0.3332	0.0989	3.3706	8e–04
Fast haplotype	0.3240	0.0996	3.2527	0.0013

Table A2.2: The effect of ethanol concentration and genotype on adult survivorship. The Fast haplotype confers higher ethanol tolerance but there is no temperature at which the T192 mutation, alone or in context of the haplotype, is deleterious. Tables A and B show summary statistics from best-fit logit models for larval ethanol survivorship at 22ª and 27º, respectively.

A

term	estimate	std.error	statistic	p.value
(Intercept)	–7.4078	0.8427	–8.7906	< 0.0001
Concentration	1.2829	0.1346	9.5343	< 0.0001
K192T	–0.2288	0.3176	–0.7203	0.4721
Fast haplotype	–2.2636	0.3729	–6.0696	< 0.0001
Sex:Male	–0.6964	0.2747	–2.5352	0.0119

B

term	estimate	std.error	statistic	p.value
(Intercept)	–5.5011	0.7138	–7.7073	< 0.0001
Concentration	1.2636	0.1294	9.7643	< 0.0001
K192T	–0.1303	0.3366	–0.3872	0.6989
Fast haplotype	–1.7634	0.3426	–5.1466	< 0.0001
Sex:Male	–1.0030	0.2771	–3.6190	4e–04

Table A2.3: The effect of temperature and heat treatment on larval and adult survivorship. Summary statistics of best-fit logistic models are displayed. Interaction terms listed are those that led to a statistically significant better model-fit, as determined by an F test. Ethanol treatments were at 8.5% and 5% for larvae and adult respectively.

A

term	estimate	std.error	statistic	p.value
(Intercept)	–1.9809	0.1864	–10.6276	< 0.0001
Ethanol treatment	2.2218	0.1922	11.5612	< 0.0001
Heat: 40C – 30 min	1.0493	0.2097	5.0039	< 0.0001
Heat: 32C – 48 hrs	2.8801	0.2033	14.1682	< 0.0001
K192	–0.5085	0.0998	–5.0979	< 0.0001
Fast haplotype	–0.4808	0.1011	–4.7569	< 0.0001
Ethanol treatment x Heat: 40C – 30 min	–1.1723	0.2400	–4.8850	< 0.0001
Ethanol treatment x Heat: 32C – 48hrs	–1.2770	0.2502	–5.1033	< 0.0001

B

term	estimate	std.error	statistic	p.value
(Intercept)	–3.7805	0.7842	–4.8210	< 0.0001
Ethanol treatment	1.7221	0.7837	2.1975	0.0297
Heat: 40C – 30 min	–1.0793	1.1971	–0.9016	0.3689
Heat: 32C – 48 hrs	–1.8647	1.8063	–1.0323	0.3037
K192T	–0.0522	0.1898	–0.2749	0.7838
Fast haplotype	–1.0805	0.2118	–5.1024	< 0.0001
Sex: Male	0.2396	0.1620	1.4788	0.1415
Ethanol treatment x Heat: 40C – 30 min	1.5247	1.2165	1.2533	0.2122
Ethanol treatment x Heat: 32C - 48 hrs	5.1985	1.8191	2.8577	0.0049

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