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Review

Adaptation of enzymes to temperature: searching for basic "strategies"☆

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Abstract

The pervasive influence of temperature on biological systems necessitates a suite of temperature—compensatory adaptations that span all levels of biological organization—from behavior to fine-scale molecular structure. Beginning about 50 years ago, physiological studies conducted with whole organisms or isolated tissues, by such pioneers of comparative thermal physiology as V.Ya. Alexandrov, T.H. Bullock, F.E.J. Fry, H. Precht, C.L. Prosser, and P.F. Scholander, began to document in detail the abilities of ectothermic animals to sustain relatively similar rates of metabolic activity at widely different temperatures of adaptation or acclimation. These studies naturally led to investigation of the roles played by enzymatic proteins in metabolic temperature compensation. Peter Hochachka's laboratory became an epicenter of this new focus in comparative physiology. The studies of the enzyme lactate dehydrogenase (LDH) that he initiated as a PhD student at Duke University in the mid-1960s and continued for several years at the University of British Columbia laid much of the foundation for subsequent studies of protein adaptation to temperature. Studies of orthologs of LDH have revealed the importance of conserving kinetic properties (catalytic rate constants (k_{cat}) and Michaelis-Menten constants (K_m)) and structural stability during adaptation to temperature, and recently have identified the types of amino acid substitutions causing this adaptive variation. The roles of pH and low-molecular-mass organic solutes (osmolytes) in conserving the functional and structural properties of enzymes also have been elucidated using LDH. These studies, begun in Peter Hochachka's laboratory almost 40 years ago, have been instrumental in the development of a conceptual framework for the study of biochemical adaptation, a field whose origin can be traced largely to his creative influences. This framework emphasizes the complementary roles of three "strategies" of adaptation: (1) changes in amino acid sequence that cause adaptive variation in the kinetic properties and stabilities of proteins, (2) shifts in concentrations of proteins, which are mediated through changes in gene expression and protein turnover; and (3) changes in the milieu in which proteins function, which conserve the intrinsic properties of proteins established by their primary structure and modulate protein activity in response to physiological needs. This theoretical framework has helped guide research in adaptational biochemistry for many years and now stands poised to play a critical role in the post-genomic era, as physiologists grapple with the challenge of integrating the wealth of new data on gene sequences (genome), gene expression (transcriptome and proteome), and metabolic profiles (metabolome) into a realistic physiological context that takes into account the evolutionary histories and environmental relationships of species.

Keywords: Acclimation; Adaptation; Alphastat regulation; Lactate dehydrogenase; pH; Temperature

Contents

☆ The papers in this volume are dedicated to the memory of our friend and mentor, Peter W. Hochachka, whose intelligence, curiosity, enthusiasm, and encouragement catalyzed research in diverse areas of comparative physiology and biochemistry and taught us that following one's curiosity in science can be both productive and fun.

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1. Temperature compensation in ectotherms: an historical perspective

1.1. What allows ectotherms to avoid the "tyranny of the Arrhenius equation?"

Ectothermic organisms adapted or acclimatized to different temperatures frequently exhibit rates of metabolic activity that are much more similar than would be predicted from simple Q_{10} relationships—a process known as temperature compensation of metabolism. This phenomenon has been recognized for over a century and remains a lively topic of debate among physiologists (see Clarke, 1991, 2003). Bullock (1955) provided a masterful summary of the first five decades of study of temperature compensation in ectotherms in his review written almost 50 years ago. Data gathered on a wide variety of ectothermic animals during this early period revealed a striking degree of independence of metabolic rate from influences of ambient temperature, following evolutionary adaptation, seasonal acclimatization, or laboratory acclimation. Sir Joseph Barcroft's (1934) statement, quoted by Bullock, summarized these observations quite succinctly: "...nature has learned so to exploit the biochemical situation as to escape from the tyranny of a single application of the Arrhenius equation." How evolution has achieved this "escape" through "exploitation of the biochemical machinery" remained largely unknown at the time Bullock wrote. Despite initial attempts to elucidate the cellular mechanisms that underlie temperature compensation of metabolic activity, for instance, the pioneering studies of Precht and colleagues in Kiel (Precht et al., 1955), Bullock concluded that, "Little can be said... about the mechanism or mechanisms by which some poikilothermic animals accomplish their relative independence of temperature."

The following decade witnessed considerable progress in the analysis of biochemical mechanisms of temperature compensation, and the study of proteins, in particular, began to take on an increasingly prominent role. Precht's (1958) group provided evidence for adjustments in enzymatic activity that allowed relatively stable rates of metabolic function at different temperatures of acclimation. Another aspect of thermal physiology was also gaining increased interest at this time: the physiological mechanisms responsible for setting thermal limits to life. Enzymes were an important focus of this work as well. Studies were beginning to show that enzymes and other proteins from warm-adapted species had greater thermal stability than the same proteins from cold-adapted species (e.g., Rigby, 1968; reviewed in Alexandrov, 1977). Whether these differences in enzyme thermal stability had any cause–effect relationship to temperature-adaptive differences in enzymatic activity was not known at this time, although a close linkage between stability and function was later to provide important insights into enzymatic adaptation to temperature, as I discuss below.

By the mid-1960s, then, differences among orthologous proteins (orthologs) from differently thermally adapted species were starting to be revealed, but it still remained unclear whether temperature-compensatory shifts in enzymatic function were the result of (i) adaptive changes in enzyme concentration, (ii) genetically based differences in the kinetic properties of enzymes, e.g., in catalytic rate constants (k_{cat}), or (iii) modulation of the activities of pre-existing enzymes, e.g., by low-molecular-mass constituents of the cell or covalent modification of proteins.

1.2. Enzymatic adaptation to temperature and the origins of a conceptual framework for adaptational biochemistry

In 1965, Peter Hochachka published a landmark paper that suggested roles for each of these three potential mechanisms of enzymatic adaptation to temperature. This study, which was part of his PhD work at Duke University, examined the ways in which thermal acclimation affected the level of expression and the qualitative isozyme composition of the glycolytic enzyme lactate dehydrogenase (LDH) in different tissues of goldfish (*Carassius auratus*) (Hochachka, 1965). Peter showed that the LDH isozyme compositions of different tissues were distinct and that temperature affected the relative levels of isoform expression in tissue-specific manners. Some isoforms of LDH displayed enhanced expression in the cold, suggesting that metabolic compensation could be achieved, at least in part, by altering protein concentration. These initial studies thus suggested that, during temperature acclimation, enzymes

changed both quantitatively (=concentration) and qualitatively (=different paralogous variants (paralogs) of an enzyme were differentially expressed). This study also examined the kinetic characteristics of the LDH paralogs, notably their different abilities to bind pyruvate (as indexed by the apparent Michaelis-Menten constant (K_m^{PYR})). The kinetic studies also included investigation of the effects of pH on K_m^{PYR} and maximal reaction velocity. Although the effects of pH on kinetics were studied only at a single temperature, the profound effects of pH on K_m^{PYR} that were observed hinted that the proton might be playing a pivotal role in sustaining protein function in the face of varying temperatures.

The broad implications of this initial study of LDH function and isoform expression were clear to Peter, who summarized the state of the field and provided a clear vision of its future development in a review entitled, ‘‘Organization of metabolism during temperature compensation,’’ which was published in a symposium volume edited by Prosser (Hochachka, 1967). This is a landmark paper because it not only revealed new aspects of thermal adaptation, but also, at least implicitly, indicated that adaptation to a suite of different environmental factors might be achieved by a common set of mechanisms (‘‘strategies’’), which involve adaptive alteration of what we now know as the genome, transcriptome, proteome, and metabolome.

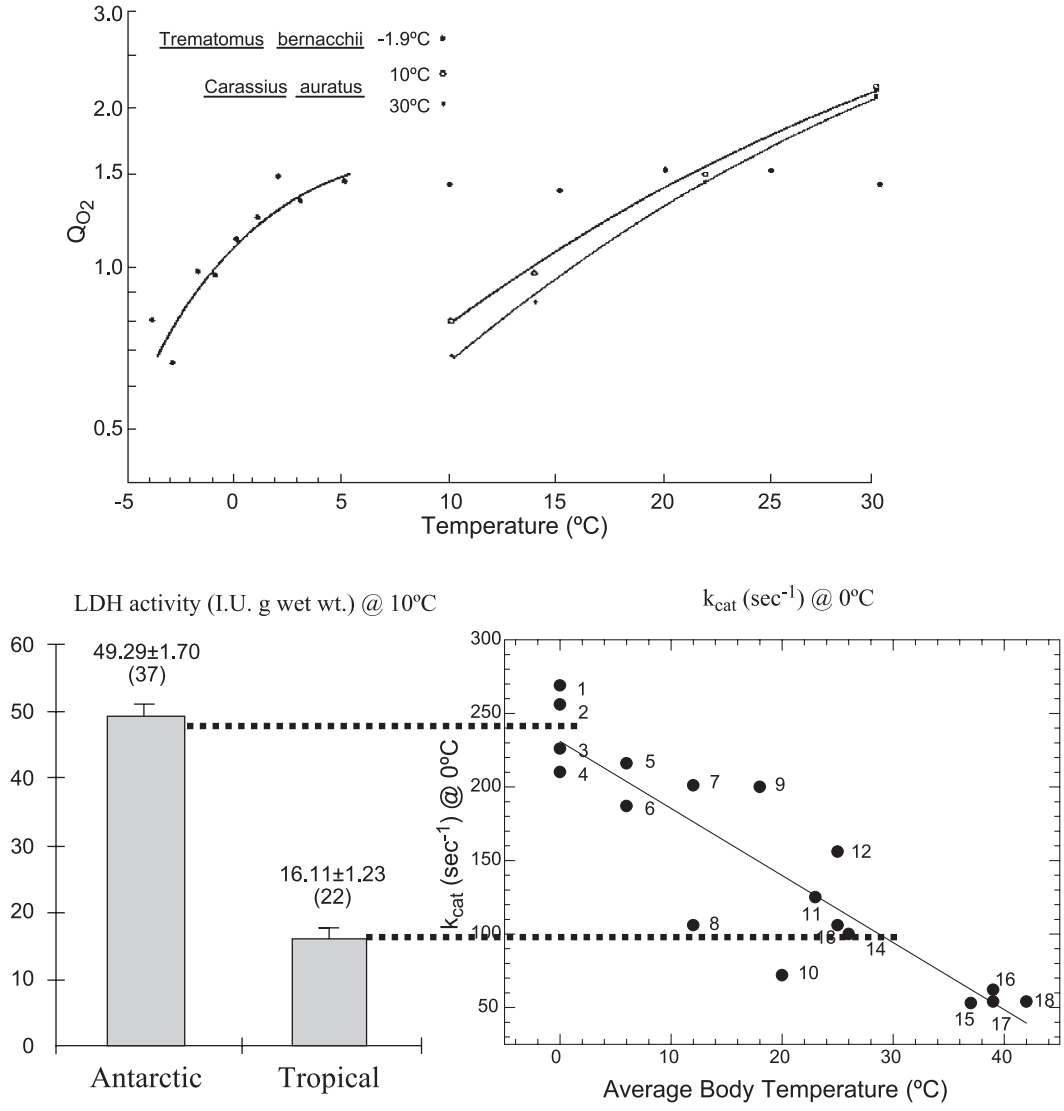

Fig. 1. Temperature compensation of brain metabolism, LDH activity normalized to wet mass, and k_{cat} values of LDH orthologs. Upper panel: oxygen consumption rates (Q_{O2}) of brain slices from the Antarctic notothenioid *Trematomus bernacchii* (Somero et al., 1968) compared to goldfish (*Carassius auratus*) (Ekberg, 1958). Figure modified after Somero et al. (1968). Lower left panel: LDH activity (International Units (IU)/g wet mass at 10 °C) in brain from Antarctic notothenioid fishes and tropical fishes (Kawall et al., 2002). Lower right panel: k_{cat} values for orthologs of A_4LDH from differently thermally adapted vertebrates (Fields and Somero, 1998). Numbers refer to different species (see Fields and Somero, 1998). Species 1–4 are Antarctic notothenioids. The dashed line connecting the LDH activity data and k_{cat} values for Antarctic and tropical species denotes the fact that the differences in wet-mass-specific activity can be largely explained by differences in k_{cat} between orthologs of Antarctic and tropical fishes.

At the time that Peter was doing his path breaking studies with LDH in differently acclimated goldfish, I was 15,000 km away studying evolutionary adaptation to extremely low temperatures in cold-adapted Antarctic notothenioid fishes. I was principally interested in three questions: First, to what extent are the metabolic rates of these—1.9 °C—adapted species compensated to the low temperatures of Antarctic waters? Second, how have many million years of evolution in a constant temperature ice-bath altered the enzymes of these fishes? Are enzymes of these cold-adapted species highly efficient catalysts that are capable of working at high rates at near-freezing temperatures? Are they extremely thermally labile due to the absence of any heat stress? Third, what are the thermal tolerance ranges of these species—and what might be responsible for causing their heat death, which Art DeVries and I found in our initial studies to occur at temperatures near 4 °C (Somero and DeVries, 1967)?

As a study system for examining questions related to metabolic compensation, I examined in vitro oxygen consumption of isolated gills and slices of brain tissue. Recent studies had demonstrated significant temperature-compensatory changes in oxygen consumption of isolated tissues following thermal acclimation (Ekberg, 1958; Kanungo and Prosser, 1960; Hochachka and Hayes, 1962), and it seemed logical to conjecture that evolutionary adaptation would be manifested at the tissue level as well. Through using isolated tissues, especially brain, in a study of evolutionary temperature compensation, one also could avoid, or at least minimize, the confounding effects of interspecific differences in locomotory habit and general activity level on metabolism. Donald Wohlschlag (1964), my graduate mentor at this time, had documented that notothenioid species differ markedly in metabolic rate as a consequence of different locomotory modes and pelagic versus demersal life styles (for an insightful analysis of this issue, see Zimmermann and Hubold, 1998). I found that oxygen consumption rates of tissues from cold-adapted notothenioids like *Trematomus bernacchii* were much higher than would be predicted from extrapolating the rates found for tissues of temperate species such as the goldfish down to McMurdo Sound temperatures of −1.9 °C (Fig. 1; Somero et al., 1968). However, as found in most analyses of metabolic compensation to temperature, compensation was not complete—a phenomenon that Peter Hochachka was subsequently to address in an insightful manner, as discussed later in this essay.

Having demonstrated a significant level of metabolic compensation to temperature in the Antarctic notothenioids, the next challenge was to determine the mechanisms that were instrumental in effecting this adaptation—the "exploitation of the biochemical machinery" that allowed "escape" from a strict adherence to the Arrhenius equation. I elected to study a mitochondrial enzyme, succinic dehydrogenase (SDH), to determine if these cold-adapted fish had enzymes that were especially well suited to drive metabolic reactions at high velocities at low temperatures. The reaction catalyzed by SDH had recently been shown to have a lower Arrhenius activation energy (E_a) in an ectotherm (frog) than in a mammal (rat) (Vroman and Brown, 1963), a finding that suggested that enzymes of cold-adapted species might be more efficient catalysts than the orthologs in warm-adapted species. Indeed, the SDH reaction of *T. bernacchii* was found to have an even lower E_a than that of the frog reaction, suggesting that E_a varied inversely with adaptation temperature (Somero et al., 1968). It appeared, then, that the "tyranny of the Arrhenius equation" mentioned by Barcroft (1934) could be circumvented, at least in part, through evolution of Arrhenius activation energies that "fit" an enzyme for function in its thermal environment. The puzzle as to why warm-adapted species benefited from "poorer" catalysts (= enzymes with high E_a values) troubled me, however, and indicated that a great deal more analysis was going to be needed before we could gain a comprehensive picture of the adaptation of enzymes to temperature. In particular, the means by which orthologous enzymes, which by definition all catalyze an identical covalent chemical reaction, could differentially reduce the activation energy barriers to their common reaction remained a serious puzzle. These issues become focal points for my intended postdoctoral research, albeit due to my isolation from the outside world, the venue for these studies remained completely up in the air.

When I returned to my home campus at Stanford University in late 1965, I arrived too late to attend the symposium on *Molecular Mechanisms of Temperature Adaptation* that C. Ladd Prosser had organized at the American Association for the Advancement of Science meeting that year. When I read the list of papers that were presented at this symposium, the title of Peter Hochachka's talk jumped out at me as being indicative of the wave of the future, a wave I hoped to catch through working in his laboratory as a postdoctoral fellow. Through a rather circuitous route, whose details are better related over a mug of beer than in the pages of a scientific journal, I arrived in Peter's laboratory in early 1967. Our mutual interest was to discover the ways in which orthologs of differently thermally adapted species differed in kinetic properties: what enzymatic traits were of pivotal importance in "fitting" a species for function in its particular thermal niche? To this end, we decided to systematically work our way along the glycolytic sequence, going from top (hexokinase) to bottom (LDH), using white muscle from differently adapted fishes as study material. My failure to isolate hexokinase from trout muscle (a set of experiments based on a remarkably naive view of fish muscle biochemistry!!) precipitated moving at once to the bottom of the pathway to examine LDH, an enzyme we knew to be abundant in muscle and, thanks to Peter's earlier work, to be a promising candidate for an effective study system. In effect, victory was snatched from the jaws of defeat, for LDH has proven to

be an excellent model system for addressing questions about enzymatic adaptation and for furthering the development of the conceptual framework we chose to call "strategies of biochemical adaptation" (Hochachka and Somero, 1973).

2. Lactate dehydrogenase: 40 years of research on a "dull" enzyme

2.1. Why "dull" is good: a Kroghian criterion for choosing a study system

Our choice of LDH as a study system also was based on the background knowledge that was beginning to become available on this enzyme due to work in other laboratories. Such a database had been building since the early 1960s, largely through the work of Nathan Kaplan's laboratory at Brandeis University (and, subsequently, at the University of California, San Diego). Studies of lactate dehydrogenase isozymes by Kaplan's group (Pesce et al., 1967) were beginning to reveal striking kinetic differences between paralogs found in heart (the H or B isozyme) and skeletal muscle (the M or A isozyme), and among orthologous variants of the A and B paralogs as well. Even at this early stage of study, then, LDH promised to be a protein that would eventually be understood in such fine detail, functionally, structurally, and evolutionarily, that our quest for a molecular analysis of adaptation would be successful. In fact, some years later, so much had become known about LDH that, at least to a non-comparative biochemist, it appeared pointless to study the enzyme any further: LDH achieved the notoriety of being the "dullest" enzyme (Ochs, 1992).

Dullness to a comparative biochemist can be a great advantage, however. If "dull" denotes a huge database on function, mechanism, and sequence, then "goldmine" might be a more appropriate word to associate with the protein. Over the next three decades, the A paralog of LDH (A_4LDH), in particular, proved to be marvelous "lab rat" protein for comparative study, one which allowed Peter and some of his academic litter to answer rather definitively the questions about enzymatic adaptation to temperature that had motivated their studies.

The LDH studies we initiated in Peter's new laboratory at the University of British Columbia in 1967 extended the studies that he had carried out with goldfish LDH by shifting focus from paralogous variants within a species to orthologous variants from species evolutionarily adapted to widely different thermal conditions. In this effort we were helped by the fact that Peter and I shared the at times questionable habit of returning home from a field site with ice chests full of dead fish. I had returned from McMurdo Sound with a collection of tissues of notothenioids and Peter had just returned from an expedition to the Amazon with tissues from a tropical lungfish. These "souvenirs" of our travels allowed us to have at our disposal LDHs from fishes whose body temperatures spanned an almost 35 °C range of temperatures, which afforded us an excellent opportunity for determining the ways in which orthologous proteins differed between "cold" and "hot" species of vertebrates.

We soon discovered that these orthologs differed in several ways that appeared to be temperature-adaptive (Hochachka and Somero, 1968). First, as shown earlier for SDH (Somero et al., 1968), the activation energies of the A_4LDH reactions were lowest in the most cold-adapted species, e.g., the notothenioid *T. bernacchii*, and highest in the most warm-adapted species, the lungfish *Lepidosiren paradoxa*. These data suggested that catalytic rate constants (k_{cat}), the rate at which substrate is converted to product normalized per active site, were inversely related to evolutionary adaptation temperature, although quantitative resolution of this question remained to be done (see below). Second, the effect of measurement temperature on substrate (pyruvate) binding (K_m^{PYR}) was strong for all orthologs, yet it appeared that intrinsic differences among orthologs in affinity for pyruvate led to a conservation of binding ability at physiological temperatures. Thus, these early studies of LDH showed that compensation to temperature meant not only a stabilization of reaction rates (k_{cat} adaptations), but also stabilization in the regulatory properties of the enzyme (K_m), such that the ability to modulate rates of catalysis in response to physiological signals was retained at different temperatures. Unfortunately, the high degree of interspecific conservation in K_m^{PYR} could not be gleaned from these initial studies because a constant pH was used in assays at all temperatures. Subsequent studies would show the importance of temperature–pH–K_m^{PYR} interactions and provide a more integrated view of protein adaptation to temperature, as discussed later (Hochachka and Lewis, 1971; Wilson, 1977; Yancey and Somero, 1978; Somero, 1995).

The molecular mechanisms underlying adaptive changes in k_{cat} and K_m^{PYR} could not be determined from these early kinetic studies. However, simple logic indicated something important about the locus of adaptive change. Thus, because binding and catalysis were likely to require identical active site structures in all orthologs, the adaptive variation in kinetic properties must be due to changes elsewhere in the enzyme. Only with an understanding of the dynamics of LDH structure, as provided by work of several groups in the 1990s (Dunn et al., 1991; Gerstein and Chothia, 1991), could this mystery be solved. A second puzzle remaining from these early studies of A_4LDH was the role of shifts in enzyme concentration in metabolic compensation: even though variation in E_a suggested that inherent differences in catalytic efficiency made an important contribution to metabolic compensation to temperature, the relative importance of compensatory changes in enzyme concentration, on the one hand, and in catalytic efficiency, on the other, remained unclear until

levels of LDH activity in tissues could be calibrated with accurate estimates of k_{cat}.

2.2. Thermodynamic activation parameters: enthalpy and entropy changes explain k_{cat} variation-and hint at the structural basis of adaptation in k_{cat} and K_m

Our finding that A_4LDH orthologs of cold-adapted species are relatively more effective than orthologs of warm-adapted species in lowering E_a values did not provide an explicit, quantitative answer to the question of how fully enzymatic rates were temperature-compensated. Arrhenius activation energies (which provide an estimate of the activation enthalpy ($\Delta H^{\ddagger}$) of a chemical reaction: $\Delta H^{\ddagger}=E_a-RT$) do not provide a quantitative measure of differences in reaction rate, because the rate of conversion of substrate to product (k_{cat}) is determined by the activation free energy ($\Delta G^{\ddagger}$), which in turn is determined by both the activation enthalpy and the activation entropy ($\Delta S^{\ddagger}$):

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

Therefore, to obtain a quantitative estimate of the differences in k_{cat} among orthologs of an enzyme, all three activation parameters must be determined (or k_{cat} must be measured directly with fully purified, fully active enzyme—a non-trivial task). Low et al. (1973) were the first to measure the k_{cat} and thermodynamic activation parameters for A_4LDH orthologs. They discovered that the differences in Arrhenius activation energy among orthologous reactions were significantly greater than the interspecific differences in $\Delta G^{\ddagger}$. This was a consequence of co-variation in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$: a high value for $\Delta H^{\ddagger}$ was paired with a high value for $\Delta S^{\ddagger}$, a phenomenon known as enthalpy–entropy compensation (Low and Somero, 1974). The lower $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ values for the A_4LDH reactions of cold-adapted species were paired with relatively unfavorable changes in $\Delta S^{\ddagger}$. The $\Delta S^{\ddagger}$ values for the A_4LDH reactions of the most cold-adapted species studied (halibut) averaged near -13.5 entropy units, whereas for A_4LDH reactions of mammals and birds $\Delta S^{\ddagger}$ ranged between approximately -2 and -8 entropy units. It would appear that, during the rate-limiting step in the A_4LDH reaction, the enzyme-substrate-cofactor complex of cold-adapted orthologs undergoes a relatively large increase in order, compared to the reactions catalyzed by orthologs of warm-adapted species (Low et al., 1973). By implication, the orthologs of cold-adapted species, when not bound with substrate and cofactor, exist in less-ordered conformations than orthologs of warm-adapted species, at any single temperature. Despite the relatively unfavorable $\Delta S^{\ddagger}$ of the A_4LDH reactions of cold-adapted species, $\Delta H^{\ddagger}$ dominates in setting $\Delta G^{\ddagger}$ so that these orthologs are able to catalyze the conversion of pyruvate to lactate at two to four times the rates noted for orthologs of warm-adapted mammals, birds, and reptiles, when measurements are made at a common temperature (Fields and Somero, 1998).

2.3. Temperature compensation and k_{cat} variation: quality, not quantity

The availability of data on k_{cat} values for orthologous A_4LDH reactions allows us to address quantitatively the question of how completely interspecific differences in k_{cat} account for temperature-compensatory differences in LDH activity in tissues of cold- and warm-adapted species. Fig. 1 presents three sets of data pertinent to this question: brain oxygen consumption (Somero et al., 1968), wet mass-specific LDH activity in brain (Kawall et al., 2002), and k_{cat} values for A_4LDH orthologs of differently thermally adapted vertebrates (Fields and Somero, 1998). In effect, these three data sets, gathered over a period of almost thirty years, retrace much of the historical pattern found in the study of temperature compensation of metabolic rate. As discussed earlier, brain metabolism is strongly compensated for temperature, and the compensation in oxygen consumption rate is mirrored closely by compensation in the rate of LDH activity in brain. Kawall et al. (2002) also showed that citrate synthase (CS), an enzyme linked to aerobic ATP production, manifests the same extent of temperature compensation seen for LDH, which indicates that enzymatic compensation to temperature occurs in both anaerobically- and aerobically poised pathways of ATP production. The differences in brain LDH activity between Antarctic and tropical fish are largely, if not entirely due to differences in k_{cat}, rather than to large variation in enzyme concentration. At common temperatures of measurement, the approximately 3-fold higher LDH activity in brain tissue of Antarctic fish is mirrored by an approximately 2.5 to 3-fold difference in k_{cat} between Antarctic notothenioids and warm-adapted fishes (Fields and Somero, 1998). We can conclude, then, that temperature compensation of LDH activity is brain is achieved during evolution by varying the intrinsic properties, i.e., the k_{cat}, of the enzyme, not by adjusting enzyme concentration.

Because organisms contain many thousands of types of enzymes, only a few of which have been examined in the context of adaptation to temperature, it would seem questionable to make too strong a generalization about the breadth of occurrence of temperature-compensatory modifications in k_{cat}. Many studies have reported temperature-compensatory changes in tissue enzymatic activity, but in only a few cases has it been determined whether variations in k_{cat} or enzyme concentration were behind these differences. For instance, as emphasized by Kawall et al. (2002), it is not known whether CS orthologs exhibit an adaptive pattern of variation in k_{cat} similar to that found for LDH. Thus, the "quality" versus "quantity" question remains unanswered for CS and most other proteins. However, as discussed below in the context of the structural bases of

k_{cat} and K_m alterations, there are valid reasons for conjecturing that the adaptive pattern noted with A_4-LDH may characterize many other proteins, notably those that undergo large changes in conformation during function.

It is appropriate to end this section with the reminder that thermal acclimation (or acclimatization) and evolutionary adaptation to temperature may entail different strategies for adjusting the levels of enzymatic activity in tissues. Thus, whereas evolutionary adaptation may involve alterations in protein sequence that cause compensatory adjustments in k_{cat}, acclimation may involve compensatory regulation of the concentrations of proteins in cells (Sidell, 1977; Rodnick and Sidell, 1995). The variety of proteins whose concentrations are adjusted during acclimation, as well as the mechanisms that underlie these adjustments, are beginning to be clarified, as genomic and proteomic technologies are brought to bear on these questions (Gracey and Cossins, 2003; Podrabsky and Somero, 2004).

2.4. An enduring mystery: why isn't compensation to temperature "perfect?"

Despite temperature-adaptive interspecific variation in k_{cat} and rate of oxygen consumption, temperature compensation is not complete for either trait. The apparent "failure" of ectotherms to fully compensate for the effects of temperature on rate processes has been a long-standing puzzle, one that Peter addressed in a 1988 paper (Hochachka, 1988). There, he conjectured that reduced energy costs of life at low temperature were the basis for incomplete temperature compensation. Specifically, costs of maintaining trans-membrane ion gradients, which represent a substantial fraction of ATP turnover in cells, were conjectured to be reduced at low temperatures. Other energy costs may also be reduced in the cold, e.g., those associated with repair of thermal damage to proteins through use of heat-shock proteins (Hochachka and Somero, 2002; Somero, 2002; Clarke, 2003). It is noteworthy that Antarctic notothenioid fishes appear unique among vertebrates in lacking a heat-shock response (Hofmann et al., 2000). Removal of irreversibly heat-damaged proteins from cells, through the energy-costly process of ubiquitination and proteolysis, also has been shown to be increased at higher environmental temperatures (Hofmann and Somero, 1995).

Factors other than energy costs also may underlie the incomplete metabolic temperature compensation typically found in ectotherms. Basic characteristics of protein structure and cellular organization may also impose limits on how fully the effects of temperature can be offset. As will become clear in the discussion of structure-function linkages in protein adaptation to temperature, constraints may exist to the extent to which k_{cat} values can be modified. If complete compensation in k_{cat} were to require an extremely labile enzyme structure, one that led to substantial denaturation of the enzyme at normal body temperatures, then trade-offs between stability and activity may come into play to restrict compensation. Considerations involving the "packaging" of enzymes within the cell (see Atkinson, 1969) also could be important in setting limits to compensation. Because of limitations in the solvent capacity of cellular water and in the number of binding sites with which enzymes interact in the formation of multi-protein complexes, it might be impossible to make up for k_{cat} shortfalls through simply adding more copies of most, if not all enzymes to the cell. Although changes in protein concentration are critical in many regulatory events in physiology, it does not seem possible, in principle, to fully temperature-compensate metabolism through increasing concentrations of all of the proteins of intermediary metabolism.

The full set of factors that contribute to setting limits to metabolic compensation remains to be revealed. It is one of Peter's legacies that he put this fascinating question into sharp relief, challenging us to find creative explanations for a curious "failure" in adaptation.

2.5. Linking variation in kinetics to alterations in structure: the "doors and hinges" hypothesis

As suggested above, structure-function linkages-and "trade-offs" between functional capacity and enzyme stability—may be critical elements in protein adaptation to temperature. We are now in a good position to examine these linkages and, thereby, analyze in detail the fine-scale molecular adaptations that account for interspecific differences in LDH function. Linking interspecific variation in functional characteristics—k_{cat}, K_m and activation parameters—to differences in structural features of A_4LDH orthologs has required detailed knowledge of the events that comprise the LDH reaction. In particular, interpretation of these differences in functional characteristics demands that we understand the rate-limiting step in the LDH reaction, for it is at this point in the overall reaction pathway that structure-function linkages accounting for variation in k_{cat}, K_m and activation parameters must be sought.

A number of highly detailed studies of LDH function have provided the backdrop of information that has allowed temperature-adaptive differences in A_4LDH function to be interpreted in fine structural detail—to "allow reptilian scales to fall from our eyes," as Peter was fond of saying (Dunn et al., 1991; Gerstein and Chothia, 1991; Deng et al., 1994; Fields and Somero, 1998; Johns and Somero, 2004). The A_4LDH reaction can be portrayed as involving three primary events: binding of substrate and cofactor, which entails large shifts in conformation that involve breaking and forming many non-covalent ("weak") chemical bonds; a covalent chemical reaction, in which substrate and cofactor are oxidized and reduced; and release of products, which essentially involves re-

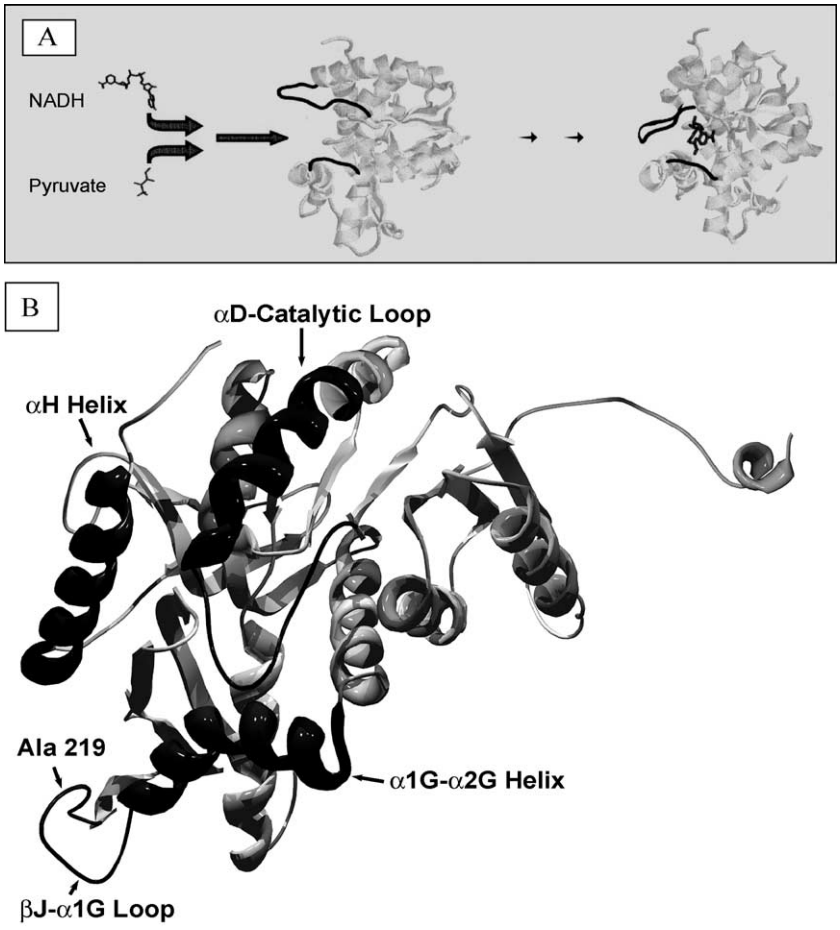

Fig. 2. Structure–function relationships in lactate dehydrogenase. (A) The catalytic conformational changes that accompany binding of substrate (pyruvate) and cofactor (NADH) by a single subunit of LDH. Note how structural elements function as "doors" that close down over the bound substrate and cofactor, establishing the catalytic vacuole in which product formation occurs. The speed of this covalent reaction is several-fold greater than the speed of the conformational changes that establish the enzyme–substrate–cofactor complex. (B) A model of a single subunit of the A_4LDH of *Chromis caudalis*, illustrating (dark shading) the components of structure that undergo the largest changes in conformation during ligand binding: the αD-catalytic loop, the α1G-α2G helix, the C-terminal αH helix, and the disordered loop connecting helix α1G with beta sheet J (βJ-α1G loop). The position of the Thr → Ala (temperate → tropical) substitution at position 219 is indicated. This model was produced by the SWISS-MODEL homology modeling program using the crystal structure of LDH-A holoenzyme from dogfish, pig and human (PDB codes 1LDM, 9LDT, and 1I10, respectively), and visualized with the Swiss-Pdb Viewer program (Guex and Peitsch, 1997). Figure modified after Johns and Somero (2004).

versing the conformational change that occurred during substrate and cofactor binding (Fig. 2, upper panel). The key fact that underlies explanations of how interspecific variation in k_{cat} and activation parameters is achieved is that the covalent chemistry is several-fold faster than the conformational changes that occur during ligand binding or release (Dunn et al., 1991). The rate-limiting conformational change accompanying binding entails large (up to approximately 15 Å) changes in the positions of several structural components, notably the alpha helix D catalytic loop, alpha helices H, 1G, and 2G, and the disordered loop that connects alpha helix 1G with beta sheet J (the α-1G-βJ loop) (Fig. 2, lower diagram). These mobile regions can be analogized to "doors" that close during ligand binding to generate the catalytic vacuole in which the covalent chemistry occurs (Dunn et al., 1991; Fields and Somero, 1998; Fields, 2001). Figuratively speaking, if these doors move on more flexible "hinges," then lower energy barriers to catalytic conformational changes will exist. Thus, cold-adapted enzymes, which often exhibit reduced thermal stability, may gain their high k_{cat} values from being more thermally labile, at least in the regions of the protein that govern the energy barriers to catalytic conformational changes (Feller and Gerday, 1997; Jaenicke, 2000).

With this structural information and the "doors and hinges" hypothesis as background, let us review the existing data on k_{cat}, K_m and LDH structure to determine (i) how amino acid substitutions simultaneously alter k_{cat}, K_m, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, i.e., how localized differences in protein structural stability are linked to differences in function; (ii) how much amino acid substitution is adequate to adapt a protein to a different range of temperatures, and (iii) what types of amino acids are substituted to achieve temperature adaptation.

2.6. Conformational microstates: a unifying concept to explain structure-function linkages in temperature adaptation

Insights into the possible trade-offs that exist in adaptation of proteins to temperature can be gleaned from analyzing the underlying basis of the co-variation in k_{cat} and K_m that has been observed in comparative studies of orthologous enzymes (Fields, 2001; Hochachka and Somero, 2002; Somero, 2003). The necessary starting point for this analysis is the dynamic nature of proteins. A protein exists in a spectrum of microstates that differ in three-dimensional configuration (see Wintrode et al., 2003). The distribution of microstates is temperature-dependent, such that increasing temperatures shift a higher fraction of the population into relatively disordered conformations, which may no longer possess the geometry needed for ligand recognition and binding (see Hochachka and Somero, 2002; Somero, 2003). From this fact it follows that (i) binding ability will be strongly influenced by temperature and (ii) the relatively flexible structures that favor high k_{cat} values ("greasier hinges" for allowing rapid movement of the "doors" that close during binding) will inevitably lead to weaker binding (larger fraction of the population of enzymes in a binding-incompetent conformation). Selection for high k_{cat} can be seen to face constraints, if the increase in structural flexibility needed to elevate k_{cat} entails too great a loss of binding ability. Conservation of binding (K_m) may thus limit the extent to which k_{cat} can be temperature compensated. Furthermore, too much structural flexibility could mean too high a likelihood of thermal inactivation of the protein.

Another insight that comes from the microstate model of protein structure concerns the covariation in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ that is observed among orthologous reactions (Low et al., 1973; Low and Somero, 1974). The more flexible structures of cold-adapted orthologs will, at a common temperature of measurement, exist in relatively more disordered conformations than warm-adapted orthologs (Hochachka and Somero, 2002; Somero, 2003). During binding, then, the cold-adapted orthologs will undergo a relatively large increase in order; $\Delta S^{\ddagger}$ will be less favorable than for warm-adapted orthologs. However, the less rigid structures of the cold-adapted orthologs will change conformation with lower enthalpy changes; $\Delta H^{\ddagger}$ will be more favorable. And, as discussed above, because interspecific differences in $\Delta H^{\ddagger}$ are greater than differences in the term $-T\Delta S^{\ddagger}$, the $\Delta G^{\ddagger}$ of the reaction catalyzed by a cold-adapted protein will be lower than that of a warm-adapted ortholog.

2.7. Adaptive changes in amino acid sequence: how many are needed, where do they occur, and what types of swaps cause adaptation?

Recent studies of orthologs of A_4LDH have provided answers to this three-part question. As our primary study system, we have used A_4LDH orthologs of confamilial and congeneric fishes. Because these orthologs exhibit only minor differences in sequence, selectively important amino acid substitutions can be discerned against a background in which few, if any, substitutions not linked to adaptation in function are found (Holland et al., 1997; Fields and Somero, 1998; Fields, 2001; Johns and Somero, 2004). These studies have shown that only one to a few amino acid substitutions is

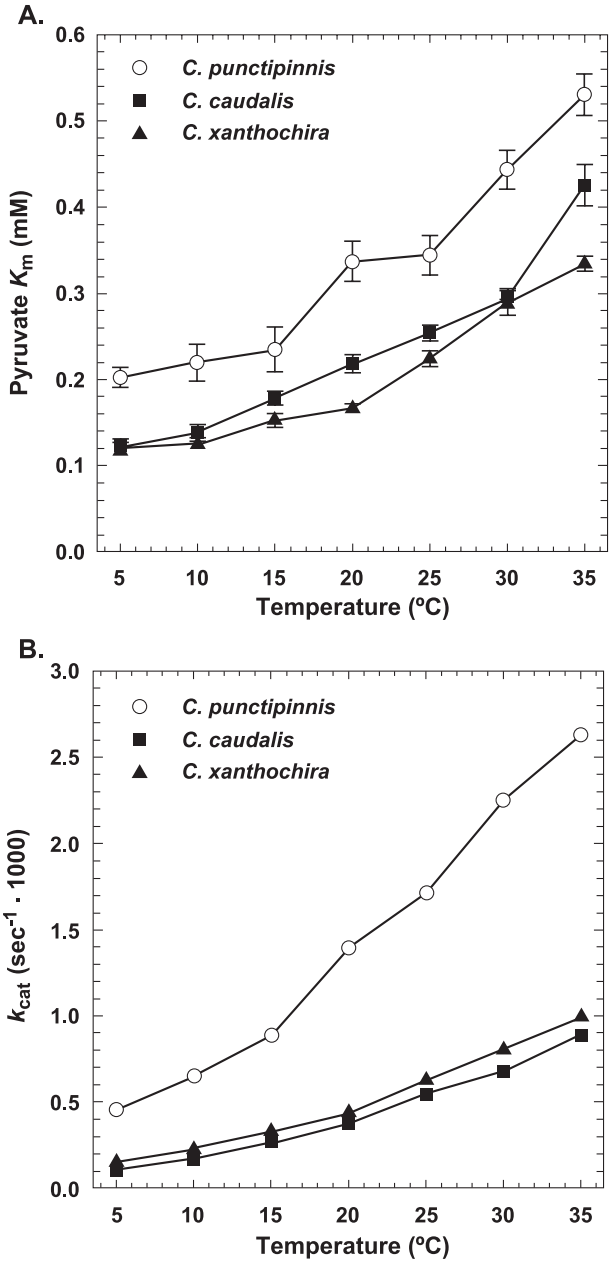

Fig. 3. Kinetic parameters, K_m^{PYR} and k_{cat}, for A_4-LDH orthologs of three congeneric damselfish of the genus *Chromis*: the temperate *Chromis punctipinnis* (○) (temperature range approximately 10–22 °C) and the two tropical species, *Chromis caudalis* (■) and *Chromis xanthochira* (▲) (temperature ranges 29 ± 1 °C). (A) K_m^{PYR}. (B) k_{cat}. Figure modified after Johns and Somero (2004).

sufficient to achieve adaptation to temperature. Thus, in a protein whose subunits comprise approximately 330 amino acid residues, less than 1% of the sequence needs to be altered to achieve adaptation to a new range of temperatures.

The sites within LDH structure where adaptive variation seems most common appear to be localized in regions that undergo large changes in conformation during the rate-limiting step, binding. As conjectured by the "doors and hinges" hypothesis, these sites typically are within alpha helices H, 1G, and 2G, and in the disordered loop that connects alpha helix 1G with beta sheet J (Fig. 2, lower diagram).

The specific amino acid substitutions that cause adaptive variation in kinetic properties through altering the conformational mobility of these regions are now being investigated using site-directed mutagenesis. An example of the type of change that is important has been found in recent studies of A_4LDH orthologs of damselfish (Johns and Somero, 2004). The basis of the interspecific differences in K_m^{PYR} and k_{cat} between LDH-A orthologs of a temperate species *Chromis punctipinnis* and two tropical congeners, *C. caudalis* and *C. xanthochira* (Fig. 3) were studied using site-directed mutagenesis of the gene encoding *C. punctipinnis* LDH-A. A single amino acid substitution at position 219 within the α-1G-βJ loop, the replacement of a threonine with an alanine, was sufficient to reduce the K_m^{PYR} of the temperate ortholog to the value noted for the orthologs of the two tropical species (k_{cat} determinations were not performed on the cloned enzymes) (Johns and Somero, 2004). The mechanism underlying this effect was conjectured to be the difference in polarity or hydrophilicity of the two residues: the more hydrophilic threonine residue was proposed to be surrounded by more water molecules, which favors a reduced interaction between the α-1G-βJ loop and the rest of the molecule. Because movement of this region of the enzyme is thought to be rate limiting (Dunn et al., 1991), this single substitution could be responsible for the higher k_{cat} and higher K_m^{PYR} of the temperate ortholog. Studies of orthologs from other species support the hypothesis that variations in sequence within the regions of the enzyme with high mobility (or in regions which affect the mobility of the "doors" that close during binding) underlie the temperature-adaptive differences in k_{cat} and K_m^{PYR} (Holland et al., 1997; Fields and Somero, 1998; Fields, 2001).

3. Temperature, pH, and proteins: creating an integrated perspective on biochemical adaptation

An uncertainty that always lurks in the background of studies performed with purified proteins in simple buffer systems concerns the fact that what is observed in vitro might deviate in important manners from what takes place in vivo. A good example of the importance of doing biochemical studies in physiologically realistic solutions is found in the context of temperature-dependent changes in intracellular pH (pH_i). As early as the mid-1920s, physiologists had discovered that pH of blood varies significantly with body temperature (for review, see Cameron, 1989). However, as Cameron (1989) emphasized in his review, "For a long time the information about how pH varied in animals as temperature changed did not seem to be disseminated among biochemists, so studies of the temperature effects on enzyme function were usually conducted at constant pH." Peter Hochachka, however, was well aware of the need for biochemists to think *biologically*: biochemical experiments should be designed to reflect the reality of the intracellular microenvironment of protein function. Thus, it is not surprising that work begun in his laboratory provided much of the founding evidence for the importance of the third "strategy" of biochemical adaptation mentioned earlier in this essay: the role played by changes in the intracellular milieu.

Studies of LDH again proved to be pivotal: temperature-dependent changes in pH were shown to play a key role in conserving the ability of the enzyme to bind pyruvate over a wide range of temperatures. The sharp dependence of K_m^{PYR} on pH shown in Peter's 1965 paper, when viewed in the light of the increasing evidence coming into the literature at that time for pervasive shifts in extracellular and intracellular pH (pH_i) with changes in body temperature (for review, see Reeves, 1977), prompted study of temperature–pH–K_m^{PYR} interactions for this enzyme (Hochachka and Lewis, 1971). This 1971 paper was an important extension of the 1965 study, for it showed the importance of proton activity in governing the thermal response of this enzyme. Through temperature-dependent variation in pH_i, K_m^{PYR} was less perturbed by changes in temperature than found under conditions of constant pH. Subsequent studies by Wilson (1977) and Yancey and Somero (1978) extended this analysis to other species and showed that the high degree of conservation of K_m found among differently thermally adapted species was the result of adaptive variation in the intrinsic binding ability of the LDH orthologs and variation in intracellular pH that results from alphastat regulation (Reeves, 1977).

In summary, study of the "micromolecular" strategy of adaptation (Hochachka and Somero, 2002), whereby adaptive changes in the composition of the intracellular solution play an important role in sustaining macromolecular function and structure in the face of physical (temperature and pressure) and chemical perturbation, was catalyzed by these early studies of LDH–pH–temperature interactions. These studies not only helped to identify the characteristics of proteins that require conservation at different temperatures, but revealed that these important conservative adaptations were a joint effect of changes in macromolecules (protein sequence) and micromolecules (H^+). The subsequent studies of other micromolecular

adaptations, most notably those involving organic osmolytes in diverse water-stressed species (Yancey et al., 1982; Fields et al., 2001; Hochachka and Somero, 2002) or deep-sea animals (Gillett et al., 1997; Kelly and Yancey, 1999; Yancey et al., 2001, 2002) sprung in large measure from the early work performed on interactions between the lowly proton and the world's dullest enzyme.

4. The segue from temperature to oxygen

In his 1967 review, Peter Hochachka presented data on temperature-dependent shifts in isozyme patterns of LDH in several species of fish. These data suggested that, within a species, differential gene expression at different ambient temperatures might play an important role in sustaining metabolic function. In this same paper, a model for temperature-dependent gene expression was presented that explained the observed shifts in LDH isozyme composition. Looking back at this early work with the wisdom of hindsight, it now appears that temperature-induced shifts in expression of the different LDH-encoding genes are more likely to be an adaptation to variations in oxygen availability than temperature per se. Recent studies of acclimation to hypoxia in euryoxic fish, using cDNA microarrays (Gracey et al., 2001), confirm that changes in oxygen availability elicit shifts in expression of LDH-encoding genes that mirror the effects found by Peter in his studies done in the late 1960s. The increased availability of oxygen at low temperatures allows a more aerobic poise to ATP production in fish, and the shifts in LDH isozyme patterns can be interpreted in this context. In fact, this conclusion is implicit in the analysis of Peter's 1967 review, which was focused on the metabolic re-organizations that accompany acclimation to different temperatures. Also evident in this 1967 review is Peter's growing interest in adaptation to oxygen availability, a topic that he soon placed center-stage in his research program and investigated, with widely different species in a vast range of habitats, during the following three decades of his career (see the essay by Ken Storey, 2004, in this volume). Thus, although adaptation of enzymes to temperature continued to be examined in his laboratory for a few more years, notably by his two students John Baldwin (Baldwin and Hochachka, 1969; Baldwin, 1971) and Tom Moon (Moon and Hochachka, 1971), the transition to an emphasis on adaptations to oxygen was well underway. The contributions of this new research program were enormous—as several papers in this volume attest. That much of this research, which involved everything from oysters to seals to turtles to humans, can be traced back to studies of thermal acclimation in goldfish LDH illustrates the remarkable way in which comparative physiology and biochemistry can evolve, when guided by a creative and exuberant mind that always managed to prod us—in its uniquely up-beat fashion—to be scientifically adventurous and enjoy our lives in science.

Acknowledgements

In common with the other authors of this memorial volume, I acknowledge the unique and catalytic contributions that Peter Hochachka made to our fields of study and to our individual careers. It is hoped that our words will reflect clearly his contributions to our lives and the gratitude we feel for his stimulation and guidance. Portions of these studies were supported by National Science Foundation grant IBN-0133184.

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