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REVIEW

Nongenetic Variation, Genetic-Environmental Interactions and Altered Gene Expression. I. Temperature, Photoperiod, Diet, pH and Sex-Related Effects

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ABSTRACT. The use of protein electrophoretic data for determining the relationships among species or populations is widespread and generally accepted. However, many confounding factors may alter the results of an electrophoretic study in such a way as to allow erroneous conclusions to be drawn in taxonomic, systematic or population studies. Such variables as temperature, photoperiod, salinity, pH and diet have been shown to influence enzymes and proteins both quantitatively and qualitatively. Production of distinct "cold" and "warm" isozymes or "seasonal" isozymes have been found in a variety of organisms. The factors that are or may be responsible for the appearance of these isozymes is discussed. Most studies that have demonstrated some apparent form of environmentally induced genetic expression have not determined the mechanisms responsible. However, proteolytic modification has been shown to produce seasonal isozymes of fructose 1,6-bisphosphatase in rabbit liver and may account for other seasonal isozymes. Acclimating organisms to various conditions may actually allow detection of cryptic genetic variation and provide valuable data. There are many aspects to consider in designing acclimation experiments, and the conditions used will vary according to the aim of the research. Polyploidy may contribute to the genesis of environmentally regulated isozymes. A review of this literature follows with additional hypotheses and conclusions. Recommendations are given for the resolution of real and potential problems. comp biochem physiol 117A;1:11–66, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Acclimation, heterogeneity, genetic polymorphism, polyploidy, voucher, inducible isozymes, constitutive isozymes, acetylcholinesterase

INTRODUCTION

Protein electrophoretic techniques have been and continue to be widely used and accepted tools in systematic and population studies of vertebrates and invertebrates (14,55,132,238,305,473,491,505), although methods of data analysis are still debated (55,327). Literature on the prevalence of biochemical systematic studies and methodologies is quite extensive (56,199,377,422,498). However, there are many factors that can affect the results of an electrophoretic study. Serious attention to known problems with electrophoresis has been lacking in the past, but some investigators have expressed concern. Johnson (237) cautioned against assuming that multiple electrophoretic bands each represent a distinct gene product and cited numerous causes of nongenetic variation. Murphy *et al.* (329) discussed some causes of post-translational modifications (PTMs; both genetic and nongenetic), cautioned against their occurrence and suggested ways of avoiding and detecting such variation. Allendorf and Phelps (8) and Kornfield (266) also mentioned briefly several causes of nongenetic variation. Noltmann (333) stated: "The simple observation that activity staining produces multiple bands after electrophoresis on agar gel is not sufficient evidence to postulate genetically determined isozymes!" Latner and Skillen (273) also noted that "Proof of the existence of isoenzymes can be extremely difficult in the absence of any pointers to molecular structure, since the activities of a number of different enzymes may overlap, as for example with the esterases. The mere finding of multiple bands of activity after any kind of electrophoresis is therefore not sufficient proof." Watts (491) discussed a variety of environmental influences on *in vivo* enzyme levels and concluded that "Such variation can be the undoing of those who prospect for a simple biochemical taxonomy."

Some investigators briefly mentioned the presence of "extra" proteins which are PTMs or nongenetic in origin (472). Richardson *et al.* (377) detailed nongenetic variation thoroughly and included many examples of nongenetic and un-

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interpretable variation on actual zymograms. The possible ramifications of such variation with regard to systematics has rarely been considered (36,50,328,477). Brush (50) stated: "Direct studies on the nature and occurrence of nongenetic changes in samples and attempts to assess fully the influence they might have on any systematic statements are not common, but appear critical as long as simple mobility is used as a criteria of similarity." Kelley and Adams (251), Oxford (338) and McGovern and Tracy (310,311) addressed how these changes may affect interpretations and conclusions. Oxford (338) provided one of the most extensive discussions of nongenetic variation and its potential impacts:

"For example, if two electrophoretically distinct isozymes are occasionally produced from a single primary product, näive interpretation of the zymogram may lead to (a) an overestimate of the number of loci revealed and/or (b) an overestimate of the number of alleles at a locus and/or (c) a false estimate of the heterozygosity at the locus. Confusion is compounded when the modified zones mimic zones that are the primary products of other alleles at the same locus or even alleles at completely different loci. . . . This may be especially misleading when the modifications do not always occur in all individuals. . . . it is obviously important to determine the conditions under which secondary isozymes are produced and to take steps to ensure that they do not occur under the electrophoretic procedures employed."

McGovern and Tracy (311) stated: "This study and others suggest that conclusions about the genetic composition of populations based on electrophoretic analyses must be supported by evidence that the protein systems studied do not show lability within individuals under abiotic or biotic stress. . . . Indeed, studies of population regulation mechanisms, of interdemic differences in behavior, of phylogeny and systematics, and of other topics should justify scientific interpretations of electrophoretic analyses in light of data showing that electromorphs of some proteins can be physiologically plastic."

Hazel and Prosser (189) similarly concluded: "Thus, physiologically speaking, the phenotype is a dynamic rather than a static quality of an organism and the expressed phenotype may be dramatically influenced (within prescribed limits established by the genotype) by prevailing factors in the physical environment."

Gill (150) stated: "If non-genetic variation can be shown to be a common phenomenon, a genetic analysis may be subject to bias if animals are not maintained under closely controlled laboratory conditions . . ." van Tets and Cowan (477) also suggested keeping all animals under identical conditions before protein extraction. However, even if all specimens are held under controlled conditions for a suitable time period, some nongenetic variation may still be present [e.g., (407)]. In a discussion of the specific distinction between *Anguilla anguilla* and *A. rostrata*, Williams and Koehn (510) remarked that ". . . no attempt has yet been made to assess geographic variation in these phenotypes. Another difficulty with drawing this conclusion is that we do not know of a genetic basis for the phenotypic differences described. One electrophoretic band does not necessarily represent a genetic difference, particularly when we are ignorant of the specific nature of the studied protein. Such a difference could be due to environmental or developmental influences."

The number of isozymes or allozymes (= multiple staining bands on a gel) expressed has been shown to be affected by temperature, diet, pH, photoperiod, sex, female reproductive state, post-translational changes, sample processing procedures, experimental methodology, storage time, pollution, disease, parasites and other stressors [this publication; reviews in (349,350)]. Booke (38) published an early review of both qualitative and quantitative variations in fish serum proteins and cited studies demonstrating seasonal, diet-related, disease-related and sexual variation in serum albumins, globulins and other general proteins. Johnston (240), Hochachka and Somero (214) and Prosser (360) discussed in detail several cases (also cited herein) of qualitative and quantitative enzyme and protein changes but did not emphasize the problems associated with such phenomena. Misinterpretation of nongenetic or inducible variation as true, genetic polymorphisms due to the abovementioned environmental or endogenous factors and the effects the changes will have with regard to systematic studies have not been treated thoroughly. The purpose of this review is to examine known causes of artifacts in electrophoretic studies and discuss methods that will help avoid such problems. In many of the studies mentioned in the following sections, the specific mechanism(s) responsible for the observed variation have not been elucidated. Often, the explanation provided by the original investigators will be cited; their explanation may or may not be the cause of the observed variation. Future research that defines the causes would be of great interest and usefulness; however, the variation observed by these investigators is significant to point out regardless of whether the true cause is known.

TERMINOLOGY

Names of North American fishes follow Robins *et al.* (381), mollusks (except *Corbicula*) follow Turgeon *et al.* (464) and names of other taxa follow those used in the cited reference. Genetic nomenclature follows Shaklee *et al.* (404). The term acclimation typically refers to the control of one variable in a laboratory, whereas acclimatization means the multivariable conditions an organism was exposed to in its natural environment (207). Rome *et al.* (385) reviewed the use of the term acclimation; they identified three definitions of acclimation that have been used in the literature previously and proposed a fourth definition that was more restrictive. Within this paper, the term acclimation does not imply that any compensation has occurred but rather indicates

that experimental organisms have been held under controlled conditions (e.g., cold-acclimated or 20°C acclimation temperature). Constitutive refers to an enzyme or protein that is constantly synthesized/present, whereas inducible enzymes or proteins can be repressed/induced under the proper conditions. Abbreviations used in this paper are as follows: post-translational modification (PTM), chromatography (CHR), juvenile hormone (JH), apparent Michaelis-Menten constant (K_m), activation energy (E_a), critical thermal maxima (CTMax), cyclic adenosine monophosphate (cAMP), isoelectric focusing (IEF), isoelectric point (pI), polyacrylamide gel electrophoresis (PAGE), starch gel electrophoresis (SGE), cellulose acetate gel electrophoresis (CAGE), spectrophotometric assay (SPA), densitometry (DST). Enzyme and protein abbreviations generally follow Shaklee *et al.* (404) and Murphy *et al.* (329) and are as follows: antifreeze glycoproteins (AFGP); aspartate aminotransferase (AAT), EC 2.6.1.1; alanine aminotransferase (ALAT), EC 2.6.1.2; acetylcholinesterase (AChE), EC 3.1.1.7; alcohol dehydrogenase (ADH), EC 1.1.1.1; acid phosphatase (ACP), EC 3.1.3.2; alkaline phosphatase (ALP), EC 3.1.3.1; adenylate kinase (AK), EC 2.7.4.3; α-amylase (AMY), EC 3.2.1.1; adenosinetriphosphatase (ATPase), EC 3.6.1.3; butyrylcholinesterase (BChE), EC 3.1.1.8; catalase (CAT), EC 1.11.1.6; citrate synthase (CS), EC 4.1.3.7; creatine kinase (CK), EC 2.7.3.2; cholinesterase (ChE) (AChE and/or BChE), EC 3.1.1._; cytosol aminopeptidase (CAP), EC 3.4.11.1 (formerly leucine aminopeptidase, LAP); cytochrome *c* (CYT *c*), EC 4.4.3._; cytochrome oxidase (CYTOX), EC 1.9.3.1; enolase (ENO), EC 4.2.1.11; esterase (EST), EC 3.1.1._; fructose bisphosphatase (FBP), EC 3.1.3.11; fructose-bisphosphate aldolase (FBALD), EC 4.1.2.13; glucose dehydrogenase (GDH), EC 1.1.1.47; glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49; glucokinase (GK), EC 2.7.1.2; glucose-6-phosphate isomerase (GPI), EC 5.3.1.9; α-glucosidase (aGLU), EC 3.2.1.20; glutathione peroxidase (GPX), EC 1.11.1.9; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), EC 1.2.1.12; glycerol-3-phosphate dehydrogenase (G3PDH), EC 1.1.1.8; glycogen phosphorylase (GPase), EC 2.4.1.1; heat shock protein (HSP); hemoglobin (HB); hexokinase (HK), EC 2.7.1.1; isocitrate dehydrogenase, $NADP^+$ (IDHP), cytosolic IDHP (sIDHP), mitochondrial IDHP (mIDHP), EC 1.1.1.42; lactate dehydrogenase (LDH), "muscle-type" LDH (LDH A, also listed as M-LDH by some authors), hear-type" LDH (LDH B, also listed as H-LDH by some authors), EC 1.1.1.27; malate dehydrogenase (MDH) and cytosolic malate dehydrogenase (sMDH), EC 1.1.1.37; malic enzyme, $NADP^+$ (MEP) and cytosolic malic enzyme (sMEP), EC 1.1.1.40; mixed-function oxidases (MFO), EC 1.14.14.1; myosin light chain (MLC); ribulosebisphosphate carboxylase (Rubisco), EC 4.1.1.39; peroxidase (PER), EC 1.11.1.7; phospho*enol*pyruvate carboxylase (PEPCX), EC 4.1.1.31; phospho*enol*pyruvate carboxykinase (PEPCK), EC 4.1.1.32; 6-phosphofructokinase (PFK), EC 2.7.1.11; phosphogluconate dehydrogenase (PGDH), EC 1.1.1.44; phosphoglucomutase (PGM), EC 5.4.2.2; protein, general (PROT); pyruvate dehydrogenase (PDH), EC 1.2.4.1; pyruvate kinase (PK), EC 2.7.1.40; superoxide dismutase (SOD), EC 1.15.1.1; succinate dehydrogenase (SUDH), EC 1.3.99.1; transferrin, TF; triose-phosphate isomerase (TPI), EC 5.3.1.1; tyrosine aminotransferase (TAT), EC 2.6.1.5; xanthine oxidase (XO), EC 1.1.3.22.

THERMALLY, PHOTO- AND OXYGEN-INDUCED ALTERATIONS

A number of studies have dealt with the reduction, alteration or complete replacement of enzymes as a result of temperature changes experienced by poikilothermic or heterothermic organisms, many of which occupy wide-ranging thermal habitats. Studies conducted by Baldwin and Hochachka (17), Moon and Hochachka (321,322), Kent and Hart (253) and others have indicated that environmentally cued protein changes occur in some fish species. Hazel and Prosser (189) reviewed some of the cases discussed herein; however, they were primarily concerned with how the enzymatic changes related to thermal adaptation. Several of the studies classified in the qualitative category by Hazel and Prosser (189) actually demonstrated only quantitative variation and are so classified in this paper. Nevertheless, the review by Hazel and Prosser (189) was one of the early accounts of thermally induced quantitative and qualitative enzyme alterations. Not accounting for qualitative or quantitative, environmentally induced isozyme or allozyme changes may result in misinterpretation of electrophoretic data indicating more or less genetic similarity than actually is present; the ultimate result of this may be a phylogenetic arrangement, description of a new taxon or population distinction that is invalid.

Few investigators have acclimated specimens before conducting biochemical studies that were not directly concerned with acclimation (1,76,151,315,478,523,524,526). Borowsky (40) encountered a great deal of variation in water parameters such as temperature, pH and dissolved oxygen even among adjacent aquaria; he suspected that these wide-ranging environmental factors were at least partially responsible for observed phenotypic (physical) differences in *Xiphophorus* spp. Borowsky (40) stated: "Our experiments now employ rigid control of feeding, temperature, pH, oxygen tension and photoperiod. In addition, all fish in our experiments are now maintained in chemical and visual isolation in their own tanks to avoid social effects on physiology. . . ." Merritt (315) acclimated *Pimephales promelas* to 5°C for a 4-week period to reduce variability prior to examining LDH gel patterns. Heterozygote LDH zymograms exhibited unexplained variation in some fishes that had been frozen in the field and not acclimated (315). The high levels of polymorphism reported in electrophoretic studies may be partially due to nongenetic or altered genetic variation be-

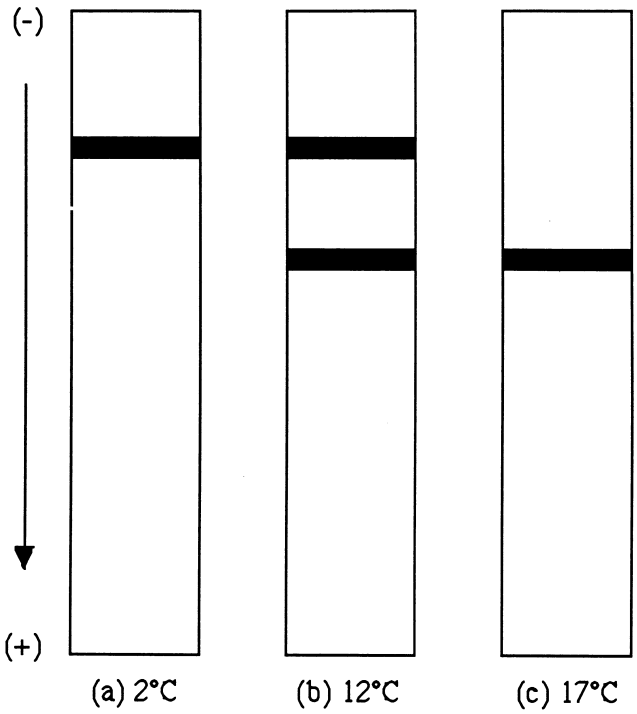

FIG. 1. Zymogram depicting AChE "isozymes" of brain extracts from rainbow trout acclimated to 2, 12 and 17°C. (From Baldwin, J.; Hochachka, P.W. Biochem. J. 116:883–887;1970, with kind permission from the Biochemical Society, London.)

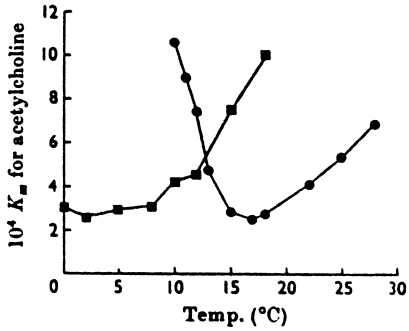

FIG. 2. K_m of AChE for acetylcholine from 2°C (■) and 17°C (●) acclimated *Oncorhynchus mykiss* brain showing minimum K_m at their respective acclimation temperatures. (From Baldwin, J.; Hochachka, P.W. Biochem. J. 116:883–887;1970, with kind permission from the Biochemical Society, London.)

cause specimens have not been held under standardized conditions but rather captured via netting or electrofishing and frozen shortly thereafter on dry ice or in liquid nitrogen.

QUALITATIVE ALTERATIONS OF PHENOTYPE

Probably the most well-known case of an environmentally induced enzyme change is that of Baldwin and Hochachka (17). Baldwin and Hochachka (17) discovered an apparent qualitative, thermally induced isozyme change in rainbow trout (*Oncorhynchus mykiss*) brain AChE isozymes with a different form of the enzyme present depending on the ambient temperature (Fig. 1). The "cold isozyme" was present at 2°C, whereas the "warm isozyme" was present at 17°C. No distinction was made as to what temperatures the actual "switching over" occurred in the trout brain tissue; however, both AChE isozymes were present at the intermediate temperature of 12°C. Many possible explanations exist for the AChE isozymes observed by Baldwin and Hochachka (17); several are mentioned below shortly, and other possible explanations are discussed in a later paper on post-translational modifications (350).

In vivo, many forms of AChE occur in vertebrates; all forms are encoded by a single gene (389,448). All vertebrates examined thus far have only a single copy of the AChE gene (75,448). Multiple forms of AChE are the result of alternative splicing and post-translational modifications such as the addition of lipid and carbohydrate groups (415,448). A difference in sialylation is possible, and higher proportions of polysialylated brain gangliosides have been found in several fish species acclimated to cold vs warm temperatures (22), including rainbow trout (197). Sialic acids were responsible for at least some of the "polymorphisms" in herring, *Clupea harengus*, muscle EST (416). Proteolytic modification also may have been responsible for multiple AChE in some cases. Proteolytic modification of BChE tetramers resulted in several additional bands of activity revealed by nondenaturing SDS-PAGE to be monomer, dimer, trimer and tetramer (all with activity) (285). Thermally induced changes noted in *O. mykiss* brain AChE may also have been due to changes in the lipid structure of membranes or glycosyl-phosphatidylinositol anchors because some AChE are associated with membranes [(121), review in 287]. Refer to Hazel and Prosser (189), Hazel (183,184,185,186), Hazel and Williams (190) and Williams and Hazel (508,509) for examples of thermally induced membrane alterations. However, because salmonids are tetraploid and express many duplicate loci, it seems reasonable to believe they possess two functional AChE genes (see polyploidy section). The author is currently investigating some potential mechanisms that may explain the warm and cold AChE "isozymes" from rainbow trout (i.e., sialic acids or proteolytic modification).

The fact that differences in K_m exist between the cold and warm isozymes of *O. mykiss* suggests that the isozymes may be encoded by different genes (Fig. 2). The obvious differences in K_m between the cold and warm isozymes may be due, at least partially, to the use of a constant pH in the assays [see (80,432), discussion on p. 27]. Differences in K_m

have been shown to result from post-translational modifications and other factors. Phosphorylation of rabbit muscle ENO altered the K_m of both the forward and backward reactions assayed at 25°C (330), and phosphorylation of CK-B subunits reduced the K_m for phosphocreatine by ≈50% (363). Sulfydryl oxidation of PK subunits increased the K_m for phosphoenolpyruvate (15). Therefore, K_m can change due to a post-translational modification. The K_m (pyruvate) for *Salvelinus fontinalis* hepatic LDH differed between cold-acclimated (4°C) and warm-acclimated (18°C) fishes, although no electrophoretic difference (quantitative or qualitative) was observed. Besides the acclimation effect on K_m, enzyme concentration, pH and assay temperature also affected the K_m (212). Walsh and Somero (488) also observed a distinct difference in K_m values for *Gillichthys mirabilis* muscle LDH between fixed pH and realistic pH-temperature relationship. *Misgurnus fossilis* LDH A_4 from 5°C- and 18°C-acclimated fishes differed in K_m and stability, although K_m was similar for both the cold and warm LDH reactivated [after denaturation (339)] indicating that conformational changes may have been present, and the LDH refolded to similar forms. Markel (301) also suggested that different MDH isozymes were present in 8 and 18°C acclimated limpets (*Collisella limatula*) based on differences in K_m and activity (after heat treatment). Although of interest, differences in K_m should not be considered alone as proof for the existence of isozymes.

A situation similar to the trout AChE was found for PK from hibernating and nonhibernating Arctic ground squirrels, *Spermophilus* (= *Citellus*) *undulatus* (may be *S. parryii*). IEF revealed a PK isozyme with a pI of 5.7 in hibernating squirrels, whereas in nonhibernators the PK isozyme had a pI of 5.2. Both PK isozymes were present in squirrels preparing to come out of hibernation within 2–3 weeks (24,26). Comparisons of PK isozymes from muscle and liver of hibernating and nonhibernating *Myotis lucifugus* revealed qualitative changes in the isozyme patterns on PAGE (39). Seasonal FBP isozymes were also suspected in *S. undulatus* based on IEF and in temperature acclimated crabs, *Paralithodes camtschatica*, based on differences in K_m (23). FBP from summer and winter collected crabs also differed in pI (25). Behrisch *et al.* (27) described cold and warm isozymes of PFK, FBP, FBALD, TPI, PK and LDH differing in pI from ear tissue of *Lepus americanus* collected in summer and winter.

Proteolytic modification of rabbit liver FBP involves the removal of an amino terminal tryptophan-containing peptide and produces an enzyme with different catalytic properties (352). The production of "seasonal isozymes" of FBP in rabbit liver was due to a much higher level of proteolytic activity in liver extracts from winter animals compared with summer animals. Pontremoli *et al.* (351) postulated that lysosomes from winter animals may be less stable and prone to leakage of proteolytic enzymes or rupture during extraction. Other seasonal isozymes may be formed by similar processes (see Dietary-Induced Alterations). Modifications of extraction procedures or use of proteolytic inhibitors may help correct such situations (276). The seasonal isozymes of FBP in *P. camtschatica* and *C. undulatus* may also result from proteolytic modification because the region in which the proteolytic-susceptible site occurs is a highly conservative region of FBP (355), and Behrisch's (25) results appear to be concordant with such a hypothesis. Fish (*Genypterus chilensis*) liver FBP is also labile to proteolytic modification, and the enzyme's catalytic properties are also significantly altered. Modification of the purification procedure eliminated the conversion of "neutral" FBP to "alkaline" FBP (159). Chum salmon (*O. keta*) in spawning migration have much higher (three to seven times) levels of several proteolytic enzymes in white muscle compared with salmon in feeding migration (520).

Smit *et al.* (421) acclimated *Carassius auratus* to 15 and 30°C and noted qualitative differences for LDH (fewer staining fractions) in 30°C fish as opposed to 15°C fish for both red and white muscle. Hillis and Patton (200) described a qualitative seasonal change in AK isozymes from *Corbicula* sp. [cf. *fluminalis*, purple form; see (314)] captured in the Brazos River, Texas. AK-2 was present in all individuals ($n = 25$) collected in April but was completely absent from all January specimens ($n = 25$) from the same locality. A possible explanation for the additional isozyme in April specimens as opposed to January specimens is activity differences in proteolytic enzymes. Because whole bodies were examined, proteolytic enzymes were likely in the extract and their activity would probably be higher in April specimens than January specimens; thus, the additional isozyme (due to PTM) in April specimens. Freezing molluscans can result in rupture of the hepatopancreas and release of proteolytic enzymes (491); Hillis and Patton (200) did not mention if samples were frozen. A re-examination of *C.* sp. (cf. *fluminalis*) using specific tissues, excluding the digestive tract, may help explain the true nature of the variation or protease inhibitors could be used (276). However, if proteolytic enzymes were responsible, one would expect other enzymes to be affected and in *C. fluminea* as well, unless proteolytic activity is influenced seasonally in *C.* sp. (cf. *fluminalis*).

Hochachka and Lewis (211) described both warm and cold isozymes of CS from thermally acclimated *O. mykiss*; their investigation included SPA and IEF to determine the identity of the two isozymes. The isozyme from 18°C trout had a lower pI (5.05) than the 2°C trout isozyme (5.76), and the two isozymes differed kinetically as well. Further work is required to determine if the two isozymes can be separated electrophoretically and if they are the products of different genes. Thermally induced isozymes have also been noted in the cyprinid, *Semotilus atromaculatus*, for hepatic LDH and G6PDH (253); photoperiod, temperature and length of acclimation time were varied in their study. Tissues examined for LDH and G6PDH were liver, gill, muscle, heart and brain. Two additional hepatic G6PDH bands

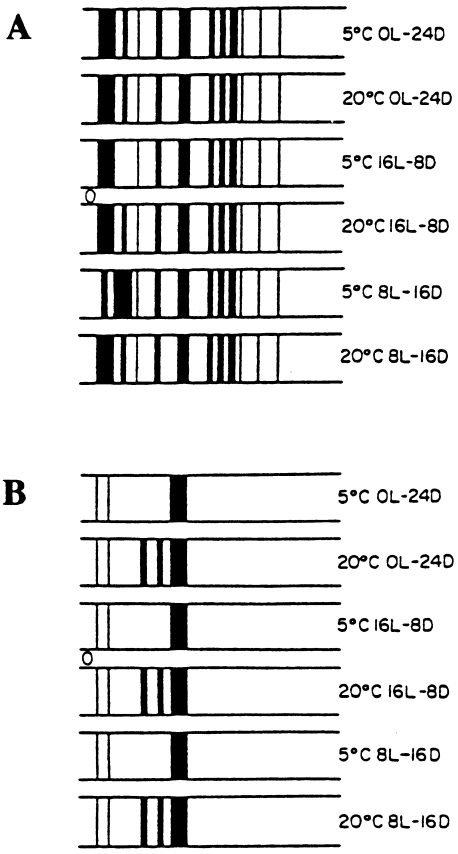

FIG. 3. Zymograms showing hepatic LDH (A) and G6PDH (B) "isozyme" expression in *Semotilus atromaculatus* under several temperature and photoperiod regimes. "O" indicates the origin, and the anode is on the right. (Reprinted from J.D.; Kent, Hart, R.G. The effect of temperature and photoperiod on isozyme induction in selected tissues of the creek chub *Semotilus atromaculatus*. Comp. Biochem. Physiol. 54B:77–80;1976, with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.)

were present in 20°C fishes as opposed to 5°C fishes, although the two bands were in some cases negligibly present at 5°C. Hepatic LDH appeared to be affected by a combination of photoperiod and temperature; qualitative changes were noted between 5 and 20°C (16L:8D) groups (Fig. 3). The synthesis of new proteins due to temperature changes has also been demonstrated in some coldwater fishes that synthesize AFGP (117, 118). Some fish species known to produce AFGP are *Pleuronectes americanus*, *Pleuronectes ferrugineus*, *Microgadus tomcod*, *Myoxocephalus aeneus*, *M. scorpius*, *Hemitripterus americanus*, *Osmerus mordax* and *Anoplarchus purpurescens* (northern populations) (118). Karr *et al.* (249) suggested that qualitative changes in cytochrome(s) P-450 were occurring among groups of *Lepomis macrochirus* acclimated to 10, 20 and 30°C; however, their kinetic data were not specific for individual isozymes or allozymes and do not demonstrate the involvement of temperature-specific isozymes. Also, if the kinetic data are examined for tests in which acclimation temperature equals assay temperature, the K_m values are very similar (i.e., not significantly different). Three additional bands of G6PDH activity appeared in 10 and 15°C acclimated *Salvelinus fontinalis* vs 4°C acclimated trout (521). Also, in a comparison of 5 and 10°C fed and starved trout, the three additional G6PDH bands appeared only in the 10°C starved fishes. The additional bands were revealed only when using a Tris–citrate, Tris–borate buffer, and storage of extracts overnight at 4°C eliminated the additional bands. The origin of the three bands was unknown and could have been epigenetic (521); however, regardless of the cause, the appearance of such staining fractions could be misleading. *Misgurnus fossilis* acclimated to 0 and 20°C differed in serum IDHP profiles. Serum from cold-acclimated fishes had only one IDHP band, whereas serum from warm-acclimated fishes had three IDHP bands (317). The difference may have been due to blood collection by means of severing the caudal peduncle, thus contaminating the blood with muscle IDHP isozymes, which were of identical mobility to the two "additional" IDHP bands in warm-acclimated fishes. The contamination would have only occurred in the warm-acclimated fishes but is still a plausible explanation [e.g., (146)].

Gill (149) reported various LDH isozymes present in *Cepaea nemoralis* during different seasons that were the result of temperature and other variables. Estivating snails possessed unique LDH isozymes. Gill (149) controlled temperature (18–20°C) and acclimated specimens for a 3-week period. Gill (150) also investigated variation in *C. nemoralis* ACP, ALP and G3PDH using PAGE. Snails were allowed an estivation period of 3 weeks and then were fed fresh carrots and lettuce every day for 7 days. ACP, ALP and G3PDH were examined each day from eight snails chosen at random. Both qualitative and quantitative variations were found for all three enzymes over the 7-day period. Possible causes of variation in ACP and ALP are discussed in a later paper on PTMs (350) and may relate to the observations of Gill (150). Marcus (299) electrophoretically analyzed four enzymes, MDH, HK, ACP and EST, in the sea urchin, *Arbacia punctulata*, and discovered a qualitative, temperature-related isozyme change in EST. Marcus (299) initially acclimated specimens to 19°C for more than 4 weeks, removed a sample of tube feet and then acclimated the still live specimens to 2°C, 19°C (controls) and 25°C for 25 days; however, the 2°C specimens died and could not be used for analysis. Additional specimens that had been acclimated to 13°C for 28 days were sampled, then acclimated to 25°C for 14 days, sampled and returned to 13°C for 1 week and then sampled again. A group originally acclimated to 19°C, then 25°C was acclimated to 13°C for 14 days and sampled, then acclimated to 25°C again for 1 week and sampled. EST patterns of 13 and 19°C urchins were similar, whereas the patterns of 25°C urchins were different; 25°C urchins possessed an additional band of EST-3 activity. The EST-3 pat-

terns of an individual urchin would change according to the acclimation temperature; however, pattern reversals required ≈14 days or longer and were not observed in individuals acclimated to the new temperature for only 7 days. An examination of Fig. 1 in Marcus (299) also seems to indicate a quantitative difference in EST-2 between 19 and 25°C. General muscle protein patterns in four populations of the crayfish, *Cambarus bartoni*, appeared to be influenced by the conductivity of stream water (465); however, the cause of the variation remains to be elucidated.

Douglas *et al.* (115) observed several qualitative and quantitative changes in polypeptides on SDS-PAGE between normoxic and anoxic turtles (*Trachemys scripta elegans*). Turtles held under anoxic conditions for 16 hr possessed a novel 19.5-kDa polypeptide in liver, increased levels of 28.6- and 79.9-kDa proteins in liver and a novel 37.5-kDa polypeptide in red skeletal muscle. A novel 32.8-kDa polypeptide appeared in turtle kidneys after recovery from anoxia. Three polypeptides were also absent from anoxic red muscle compared with normoxic controls (115). McGovern and Tracy (310,311) studied temporal changes in allele frequencies of serum TF and CAP with SGE in populations of the prairie vole, *Microtus ochrogaster*; some "electromorphs" of each protein were found to change over time within the same individual. McGovern and Tracy (310) sampled individuals upon capture and then subjected groups to various environmental regimes in the laboratory and subsequently resampled the same individuals. McGovern and Tracy (311) examined individuals from two 1-ha, outdoor plots and collected seasonal samples from individuals. Of the 137 voles sampled more than once, 40 exhibited an altered TF gel pattern, 24 had altered CAP gel patterns and 10 possessed both altered TF and CAP (311). The studies of McGovern and Tracy (310,311) clearly indicate the "instability" of at least some "electromorphs" of TF and CAP and that the "electrophoretic genotype" is not absolute in some cases. Similarly, Baker *et al.* (16) observed qualitative changes in plasma EST gel patterns between cold- and warm-acclimated rats, and they also sampled the same individuals under both conditions. The results of Baker *et al.* (16), Marcus (299), Gill (149,150), McGovern and Tracy (310,311) and Oxford (337) are far more conclusive than the other studies in that the same individuals were tested under various acclimation conditions!

Guy (170) and Thomashow (453) reviewed cold-acclimation in plants; many adaptive strategies used by plants are apparently very similar if not identical to methods of enzymatic adjustment observed in poikilothermic vertebrates. Guy (170) and Thomashow (453) cited numerous cases of various enzymatic changes associated with acclimation to cold, including both increased and decreased (quantitative) levels of some proteins and qualitative changes; several of the most noteworthy cases are mentioned in the text below, and additional cases may be found in Tables 1 and 2. Huner and Macdowall (225,226,228,229) found cold acclimation in rye to "produce" a different isozyme of Rubisco, and conformational changes in a single enzyme were implicated as the cause (227). Thomashow (453) indicated that most of the studies he cited did not conclude what had caused the observed phenomena but did mention that post-translational modification could be a cause of the observed changes rather than altered gene expression. However, Shomer-Ilan and Waisel (410) analyzed Rubisco isozymes from acclimated and nonacclimated cabbage (*Brassica oleracea*) and determined by PAGE and amino acid analysis that the isozymes from the two groups were different, suggesting an "on-off" system of isozyme induction. Kelley and Adams (251) analyzed foliage of four *Juniperus scopulorum* collected monthly for 1 year and found qualitative alterations in EST using PAGE. The changes were attributed to seasonal effects on the physiology of the trees. An interesting observation is that one of four trees expressed the additional EST isozymes for 3 months longer in summer/autumn than the other three. Therefore, the seasonal sampling would appear to have value in detecting some genetic variation, which in this case may be differences in regulatory genes. Qualitative and quantitative alterations in both MDH and PER gel patterns were noted by De Jong (107) by using three variable night/day temperature regimes at a constant photoperiod and three variable photoperiods at a constant temperature. One puzzling observation in De Jong's data is that PER band 6 activity was not expressed in any of the temperature groups (10/15, 20/25 and 30/35°C) on 8L:16D, whereas this band was present in all photoperiod groups (6, 12 and 18 light) at 20/25°C. If band 6 was present at 20/25°C and both 6- and 12-hr light, one would expect it at 20/25°C and 8-hr light. Similar results were also shown for PER bands 8, 9, 10 and 16. Refer to Thomashow (453) and Guy (170) for a more thorough treatment of cold acclimation in plants.

Photoperiod has also been shown to have a significant influence on enzyme expression; often temperature and photoperiod exhibit combined effects. Duman and DeVries (118) found that both photoperiod and temperature were involved in the disappearance of AFGP in *P. americanus* and several other species. Both photoperiod and temperature affected the composition of hepatic LDH in *S. atromaculatus* (253). Photoperiod alone was responsible for a qualitative change in muscle LDH from *Fundulus heteroclitus* (303). The reason for altered LDH in 0L:24D fish may have been stress related (371,398). Differences in diet were not responsible; feeding behavior was monitored in both 8L:16D and 0L:24D groups. CAT-2 is completely absent in dark-grown maize leaves but is expressed shortly after exposure to white light; the opposite was found for CAT-3, which declines after exposure to light until it is undetectable (394). Warner and Upadhya (489) observed qualitative differences for EST, AMY and CAP in *Citrus* spp. and for EST, CAP and PER in *Poncirus trifoliata* between plants under short and long photoperiods. Other cases of light-mediated changes in gene expression are discussed by Scan-

TABLE 1. Cases of qualitative variation in enzymes and proteins due to (directly or indirectly) differences in temperature, photoperiod, dissolved oxygen, diet or season

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s)		Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
				Fixed	Manipulated				
Acetylcholinesterase AChE; EC 3.1.1.7	*Oncorhynchus mykiss*	30–80 Pooled	B		T			(D) PAGE TA	1
Acid Phosphatase ACP; EC 3.1.3.2	*Cepaea nemoralis*	8	HP		SC			(D) PAGE	2
	Cyclonassa neritea	?	HP		T			(G) PAGE	3
	Dianthus spp.	?	ST LF		SC		Winter and summer collected tissues differed in number of staining fractions	(D) PAGE	4
Adenylate Kinase AK; EC 2.7.4.3	*Corbicula* sp. cf. *fluminalis*	25	WB		SC			SGE	5
Alkaline Phosphatase ALP; EC 3.1.3.1	*Cepaea nemoralis*	8	HP		AE			(D) PAGE	2
α-Amylase AMY; EC 3.2.1.1	*Citrus reticulata*	?	ST		P		Only one AMY band, but mobility differed between photoperiods	(H) SGE† (H) PAGE	6
	Gammarus palustris	4–10	WB	P	T D	Range of 1 day to 7 days	AMY I^c appeared at colder (4°C) vs warmer (22°C) temperatures and also when amphipods were fed amylose or glycogen.	PAGE	62
	rats (Donrye strain)	3, all ♂	L		D	Range of 1 to 6 days	Electrophoretic mobility of AMY differed between fasted and well-fed rats	CAGE	63
Antifreeze Glycoprotein AFGP	*Anoplarchus purpurescens*	?	BL S		T P			SFP SMP	7
	Hemitripterus americanus	?	BL S		T P			SFP SMP	7
	Microgadus tomcod	?	BL S		T P			SFP SMP	7
	Myoxocephalus aeneus	?	BL S		T P			SFP SMP	7
	Myoxocephalus scorpius	?	BL S		T P			SFP SMP	7
	Osmerus mordax	?	BL S		T P			SFP SMP	7
	Pleuronectes americanus	?	BL S		T P			SFP SMP	7
	Pleuronectes ferrugineus	?	BL S		T P			SFP SMP	7
Cytosol aminopeptidase CAP; EC 3.4.11.1	*Citrus reticulata* (Dancy)	?	ST	T	P	28 weeks	Three CAP under SD (8L:16D), while four bands present under LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6
	C. paradisi X *C. reticulata*	?	ST	T	P	28 weeks	One additional band under SD (8L:16D) not present under LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6

	Microtus ochrogaster	? R	BL		T, P D			SGE	44
	Microtus ochrogaster	? R	BL		SC			SGE	45
	Poncirus trifoliata	?	ST	T	P	28 weeks	Two CAP under SD (8L:16D), while three were present under LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6
Catalase Cat; EC 1.11.1.6	*Zea mays*	?	LF		P				8
	Zea mays	?	Whole Seedlings		P				8
Citrate Synthase CS; EC 4.1.3.7	*Oncorhynchus mykiss*	?	L		T			IEF SPA	9
Enolase ENO; EC 4.2.1.11	*Saccharomyces cerevisiae*	—	—		T (Heat Shock)				10
Esterase EST; EC 3.1.1._	*Arbacia punctulata*	16‡	TF		T			(H) PAGE	11
	Cepaea hortensis	? R	HP		D			(D) PAGE	12 13
	Cepaea nemoralis	? R	HP		D			(D) PAGE	13
	Citrus reticulata (Clementine)	?	ST	T	P	28 weeks	Eleven EST bands under SD (8L:16D), while only nine bands under LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6
	Citrus reticulata (Dancy)	?	ST	T	P	28 weeks	Five EST bands under SD (8L:16D), while only FOUR bands under LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6
	C. paradisi X *C. reticulata*	?	ST	T	P	28 weeks	Ten EST bands under SD (8L:16D) and LD (range of ≈11–12 hr + 4 hr), but one band unique in each	(H) SGE† (H) PAGE	6
	Cyclonassa neritea	?	M HP		T			(G) PAGE	3
	Dianthus spp.	?	ST LF		SC		Winter and summer collected tissues differed in number of staining fractions	(D) PAGE	4
	Hyalophora gloveri pupae	3–7 ♂	HL		HO	stored at 6°C for 2–4 months	2 or 3 additional EST appear following injection of juvenile hormone	PAGE	14 15
	Juniperus scopulorum	4 R	F		SC			PAGE	16
	Monacha cantiana	? R	HP		D			(D) PAGE	13
	Poncirus trifoliata	?	ST	T	P	28 weeks	Number and position of bands differed between SD (8L:16D) & LD (range of ≈11–12 hr + 4 hr)	(H) SGE† (H) PAGE	6
	Potamopyrgus jenkinsi	?	?		D			?	13
	rats (albino)	70 ♂ R	Bl S		T	8, 16, 25, 36 & 50 days	Several EST exhibited altered mobilities at cold exposure cold vs warm temperatures	SGE DST	17

TABLE 1. (*continued*)

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s)		Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
				Fixed	Manipulated				
Fructose-biphosphatase FBP; EC 3.1.3.11	*Spermophilus undulatus*‡	?	L		NH		pI = 5.7 in CA vs pI = 5.2 in WA; both present in animals 2–3 weeks prearousal from hibernation	IEF	18 19
	Lepus americanus	?	ER		SC			IEF	20
	Lepus americanus	?	L		T D		Proteolytic modification		21
	Paralithodes camtschatica	?	M		T		K_m differed between crabs at 6–9°C vs −1–1°C	SPA	18
	Paralithodes camtschatica	?	M		SC		Ca → pI = 5.2–5.3 Wa → pI = 5.8–5.9	IEF	22
Fructose-bisphosphate Aldolase FBALD; EC 4.1.2.13	*Lepus americanus*	?	ER		SC			IEF	20
Glucose-6-phosphate Dehydr genase G6PDH; EC 1.1.1.49	*Salvelinus fontinalis*	10	L	D	T D	3 weeks	3 additional cathodic bands in 10 & 15°C not present in 4°C fishes; the 3 bands were present in 10°C (fed & starved) fishes, but not in 5°C (fed & starved) or 10°C fed fishes	PAGE	23
	Semotilus atromaculatus	4	L		T			PAGE	24
Glutathione Reductase GR; EC 1.6.4.2	*Mytilus edulis*	?	MA		SC			PAGE SPA	25
Glycerol-3-Phosphate Dehydrogenase G3PDH; EC 1.1.1.8	*Cepaea nemoralis*	8	HP		AE			(D) PAGE	2
Heat Shock Protein HSP	*Eurytemora affinis*	?	WB		T	4°, 15° & 20°C heat shock	Heat shocked copepods at each temperature expressed HSP. The 20°C group expressed HSP not observed in 4° or 15°C heat shocked copepods. This may be only extreme quantitative variation.	SDS-PAGE PL	26
Hemoglobin HB	*Barbus holubi*	?	BL		SC			DST PAGE	27
	Carassius auratus	12	BL		T		2 HB @ 2°C 3 HB @ 20° & 30°C	PAGE, DST SPS	28
	Carassius auratus	?	BL	D P	T	>28 days	2 HB @ 2°C 3 HB @ 12°, 20° & 30°C	CAGE DST	29
	Carassius auratus	12	BL		T	5° & 30°C	2 HB @ 5°C 3 HB @ 30°C	DST PAGE	30
	Carassius auratus	12	BL		T	3° & 23°C	2 HB @ 3°C 3 HB @ 23°C	PAGE DST	31
	Cyprinus carpio	?	Bl		SC		2 HB from Nov.–June 3 HB from July–Nov.	PAGE	32

	Oncorhynchus mykiss	?	BL		T	12–30 weeks	*De novo* synthesis or alterations in aggregations of existing subunits	IEF	33
	Oncorhynchus mykiss	?	BL	D, P. pH T not fixed, but same for all	O	24 days	Hypoxic fish lacked HB isoforms Cl & A5	CAGE DST	34
Isocitrate Dehydrogenase IDHP; EC 1.1.1.42 $NADP^+$	*Misgurnus fossilis*	7–10	BL S	D	T		3 isozymes in 20°C fish vs 1 isozyme in 0°C fish	(D) PAGE	35
	S. namaycush x *S. fontinalis*	?	L		T			(H) SGE	36
Lactate Dehydrogenase LDH; EC 1.1.1.27	*Carassius auratus*	1	M		T			(V) SGE	37
	Cepaea nemoralis	?	HP		AE			(D) PAGE	38
	Fundulus heteroclitus	?	M		P			(V) SGE	39
	Fundulus heteroclitus	?	M		T			(V) SGE	40
	Lepus americanus	?	ER		SC			IEF	20
	Semotilus atromaculatus	4	L		T			PAGE	24
Malate Dehydrogenase MDH; EC 1.1.1.37	*Collisella limatula*	?	V	P	T D		Enzyme from 8°C vs 18°C snails differed in K_m and susceptibility to heat degradation	SPA	41
	Nicotiana tabacum	6?	LF		T P	≈2 months		PAGE	42
Myosin light chain MLC	*Cyprinus carpio*	14	M	P D	T	6^9 weeks 8° and 20°C	An additional MLC, $MLC3_c$, was present in 8°C carp and absent or only in trace amounts in 20°C carp	SDS-PAGE IEF	43
Peroxidase PER; EC 1.11.1.7	*Dianthus* spp.	?	ST LF		SC		Winter and summer collected tissues differed in number of staining fractions	(D) PAGE	4
	Neurospora crassa	—	—		T (heat shock)	1 hr		PAGE	46
	Nicotiana tabacum	6?	LF		T P			PAGE	42
	Poncirus trifoliata	?	ST	T	P	28 weeks	Two additional PER under LD (range of ≈11–12 hr + 4 hr) not present under SD (8L:16D)	(H) SGE† (H) PAGE	6
6-Phosphofructokinase PFK; EC 2.7.1.11	*Lepus americanus*	?	ER		SC			IEF	20
	Oncorhynchus mykiss	?	M		T			SPA	47
Protein, General PROT	*Bromus inermus*	—	CC		T		2 PROT appeared @ 23° vs 3°C 3 PROT appeared @ 3° vs 23°C	TDE	48
	Cambarus bartoni	36	M		conductivity		PROT patterns were related to the conductivity of streams	PAGE	61
	Cyclonassa neritea	?	M HP		T			(G) PAGE	3
	Rhodeus sericeus amarus	?	M	P	T			CAGE (D) PAGE	49

TABLE 1. (*continued*)

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s) Fixed	Variable(s) Manipulated	Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
	Secale cereale	?	Crown		T, P MP?			SDS-PAGE 2DE, IEF	50
	Secale cereale	?	LF		T	CA → 63–70 d WA → 21–28 d		SDS-PAGE IEF	51
	Spinacia oleracea	?	L		T		22 PROT appeared at CA (5°C), but not at WA (25°C)	2DE	52
	Spinacia oleracea	?	LF (chloroplast)				1 PROT appeared in CA (5°C) not present at WA (25°C)	SDS-PAGE	53
	Trachemys scripta elegans	3	L	T	DO	3 weeks pre-acclimation 16 hr. anoxia	Novel 19.5 kDa polypeptide in anoxic turtles	SDS-PAGE	54
	Trachemys scripta elegans	3	H	T	DO	3 weeks pre-acclimation 16 hr. anoxia	Novel 26.8 kDa polypeptide in anoxic turtles	SDS-PAGE	54
	Trachemys scripta elegans	3	M (red)	T	DO	3 weeks pre-acclimation 16 hr. anoxia	Novel 37.5 kDa polypeptide in anoxic turtles, and three other polypeptides were absent	SDS-PAGE	54
	Trachemys scripta elegans	3	K	T	DO	3 weeks pre-acclimation 16 hr anoxia	Novel 32.8 kDa polypeptide in turtles during post-anoxic recovery period	SDS-PAGE	54
	Triticum aestivum	?	Crown		T, P	7 day (cont.) 40 day (exp.)	4 proteins appeared & 2 disappeared in CA plants	SDS-PAGE 2DE, IEF	55
	Triticum aestivum	?	LF		T, P	7 day (cont.) 40 day (exp.)	CH plants synthesized new proteins not in WA plants	SDS-PAGE 2DE, IEF	55
	Triticum aestivum	?	Root		T, P	7 day (cont.) 40 day (exp.)	3 proteins appeared & 6 disappeared in CA plants	SDS-PAGE 2DE, IEF	55
Pyruvate Kinase PK; EC 2.7.1.40	*Spermophilus undulatus*‡	?	L		NH		pI = 5.7 in CA vs pI = 5.2 in WA; both present in animals 2–3 weeks prearousal from hibernation	IEF	56
	Lepus americanus	?	ER		SC			IEF	20
	Myotis lucifugus	5 pooled	M L		NH		Different number of bands and positions in muscle same number of bands, some in common and some different in liver	PAGE	57

	Oncorhynchus mykiss	?	M		T			SPA	47 58
Ribulose-bisphosphate Carboxylase Rubisco; EC 4.1.1.39	*Brassica oleracea*	?	LF		T	15° & 25°C	Change in electrophoretic mobility	(D) PAGE	59
	Secale cereale	?	LF		T		Change in electrophoretic mobility	PAGE, DST	60
Transferrin TF	*Microtus ochrogaster*	? R	BL		T, P D			SGE	45
	Microtus ochrogaster	? R	BL		SC			SGE	46
Triose-phosphate Isomerase TPI; EC 5.3.1.1	*Lepus americanus*	?	ER		SC			IEf	20
Troponin I TNI	*Cyprinus carpio*	14	M	P D	T	6–9 weeks 8° & 20°C	An additional "minor" band was present in 20°C carp	SDS-PAGE IEF	43

*Sixteen initially, 6 died, then 4 were added = 14.

†Methods were listed in Brewbaker *et al.* (1968).

‡May be referable to *Spermophilus parryii*.

Abbreviations: Number Examined: R, Resampled the same individuals under different conditions.

Tissues: B, Brain; BL, Blood; CC, Cell Cultures; E, Eye; ER, Ear; F, Foliage; HL, Hemolymph; HP, Hepatopancreas; I, Intestine; LF, Leaf; L, Liver; M, Muscle; MA, Mantle Tissue; P, Prosoma; S, Serum; ST, Stem; Tf, Tube Feet; V, Viscera; Wb, Whole Body.

Variables: AE, Active vs Estivating; D, Diet; HO, Juvenile Hormone Injection vs No Juvenile Hormone Injection; NH, Normothermic vs Hibernating; P, Photoperiod; SC, Seasonal Collection; T, Temperature.

Other: CA, Cold Acclimated; WA, Warm Acclimated.

Techniques: CAGE, Cellulose Acetate Gel Electrophoresis; CHR, Chromatography; CMH, Cyanmethemoglobin Procedure; DST, Densitometry; IEF, Isoelectric Focusing; IMC, Immunochemistry; MPA, Microbiuret Protein Assay; MW, Molecular Weight; PAGE, Polyacrylamide Gel Electrophoresis (D = Disc, G = Gradient Gels); PE, Paper Electrophoresis; SGE, Starch Gel Electrophoresis (H = Horizontal, V = Vertical); SFP, Serum Freezing Point; SMP, Serum Melting Point; SPA, Spectrophotometric Assay; TA, Titration Assay; TDE, Two-Dimensional Electrophresis.

References: 1, Baldwin and Hochachka, 1970; 2, Gill, 1978b; 3, Trellu *et al.*, 1978; 4, McCown *et al.*, 1969; 5, Hillis and Patton, 1982; 6, Warner and Upadhya, 1968; 7, Duman and DeVries, 1974b; 8, Scandalios, 1990; 9, Hochachka and Lewis, 1970; 10, Tida and Yahara, 1985; 11, Marcus, 1977; 12, Oxford, 1975; 13, Oxford, 1978; 14, Whitmore *et al.*, 1972; 15, Whitmore *et al.*, 1975; 16, Kelley and Adams, 1977; 17, Baker *et al.*, 1964; 18, Behrisch, 1973; 19, Behrisch, 1978; 20, Behrisch *et al.*, 1981; 21, Pontremoli *et al.*, 1973a, b, c, 1981; 22, Behrisch, 1975; 23, Yamauchi *et al.*, 1975; 24, Kent and Hart, 1976; 25, Ramos-Martinez and Rodriguez Torres, 1985; 26, Bradley *et al.*, 1988; 27, van Vuren and Hattingh, 1978; 28, Houston and Cyr, 1974; 29; Falkner and Houston, 1966; 30, Houston *et al.*, 1976; 31, Houston and Rupert, 1976; 32, Fourie and van Vuren, 1976, 33, Weber *et al.*, 1976; 34, Marinsky *et al.*, 1990; 35, Mester *et al.*, 1972; 36, Moon and Hochachka, 1971a; 37, Smit *et al.*, 1974; 38, Gill, 1978a; 39, Massaro and Booke, 1971; 40, Bolaffi and Booke, 1974; 41, Markel, 1976; 42, De Jong, 1973; 43, Crockford and Johnson, 1990; 44, McGovern and Tracy, 1981; 45, McGovern and Tracy, 1985; 46, Kapoor and Lewis, 1987; 47, Somero and Hochachka, 1971; 48, Robertson *et al.*, 1987; 49, Hauss, 1975a; 50, Uemura and Yoshida, 1984; 51, Laroche and Hopkins, 1987; 52, Guy and Haskell, 1987; 53, Guy and Haskell, 1989; 54, Douglas *et al.*, 1994; 55, Perras and Sarhan, 1989; 56, Behrisch, 1974; 57, Borgmann and Moon, 1976; 58, Somero, 1972; 59, Shomer-Ilan and Waisel, 1975; 60, Huner and Macdowall, 1976a, b; 1978; 1979a, b; 61, Turchi *et al.*, 1988; 62, Borowsky, 1984b; 63, Takeuchi *et al.*, 1975.

TABLE 2. Cases of quatitative alterations in enzymes and proteins due to (directly or indirectly) differences in temperature, photoperiod, dissolved oxygen, diet or season

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s)		Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
				Fixed	Manipulated				
Acid Phosphatase ACP; EC 3.1.3.2	*Cepaea nemoralis*	8	HP		AE			(D) PAGE	1
	Cyclonassa neritea	?	M HP		T			(G) PAGE	2
Alkaline Phosphastase ALP; EC 3.1.3.1	*Cepaea nemoralis*	8	HP		AE			(D) PAGE	1
	Salvelinus fontinalis	?	I		T			DST, SPA (V) PAGE	3
α-Amylase AMY; EC 3.2.1.1	*Gambusia holbrooki*	12–20	Gut	P	D			ELISA	4
	Gammarus palustris	5 pooled 6 samples	WB	P T	D	4 days @ 19°C	AMY I^c and AMY I^w were both quantitatively altered (one higher, one lower; amylose vs agarose diet)	PAGE DST	5
α-glucosidase aGlu; EC 3.2.1.20	*Cyclonassa neritea*	?	HP		T			PAGE	2
Calmodulin and Calmodulin-related proteins	*Arabidopsis* sp.	?	LF Stem		See text p. 18		10–100 fold increase in five mRNAs following experimental treatments	DNA	6
Catalase Cat; EC 1.11.1.6	*Crithidia luciliae thermophila*	—	whole/ cultures		T	25°C 33°C → 2 hr 37°C → 24 hr	Decrease in activity of the single CAT	IEF SPA	7
	Zea mays	?	Whole Seedlings		P				8
Cytochrome c CYT c; EC 4.4.3._	*Lepomis cyanellus*	5–6	M		T		Higher in 5° > 15° > 25°C; rates of synthesis and degradation both lowered, but degradation rate lowered more at colder temperatures	SPA	9
Cytochrome Oxidase CYTOX; EC 1.9.3.1	*Carassius auratus*	?	M	P	T	3–4 weeks		IMC	10
Cytochrome(s) *P*-450 EC 1.14.15.6	*Lepomis macrochirus*	15 pooled per group	L	D	T	>2 weeks	Fractions 1, 3 & 4 varied among the 10°, 20° & 30°C groups	SDS-PAGE DST	11
Cytosol aminopeptidase CAP; EC 3.4.11.1	*Cyclonassa neritea*	?	HP		T			(G) PAGE	2
Esterase EST; EC 3.1.1._	*Arbacia punctulata*	14*	TF		T			PAGE	12
	Cyclonassa neritea	?	M HP		T			PAGE	2
	Goniobasis cochliaris	?	whole body		T	14 day	Increased at higher temperatures: 10°, 17° & 24 °C	(H) SGE	13
	Lepomis cyanellus	?	E L	T DO				(V) SGE SPA	14

	Rhodeus sericeus amarus	?	B		T		Greater quantity of EST 1 & 3 at colder (10°C & 20°C) vs warmer (29°C) temperatures	DST (D) PAGE	15
Fructose-bisphosphate Aldolase FBALD; EC 4.1.2.13	*Euglena gracillis*	—	Whole Organism		P M			IEF	16
	Lepomis cyanellus	?	M		T			PAGE	17
	Thamnophis sirtalis parietalis	3	H	D T	P	3 weeks	Greater staining intensity in 12L:12D group than 0L:24D group	SGE	18
Glucose-6-phosphate Dehydrogenase G6PDH; EC 1.1.1.49	*Mugil cephalus*	?	L		T P			SGE SPA	19
	Rattus sp.	?	L		D		Quantitative differences noted for all 3 dimers among fasted, 2 days, then fed) groups	(D) PAGE	20
Glucosephosphate Isomerase GPI; EC 5.3.1.9	*Fundulus heteroclitus*	5–7 fish per sample time	L	S T	DO	14, 22, 28 and 35 days 1.5–2.5 ppm (exp.) 8–9 ppm (cont.)		SPA MPA (H) SGE	21
Glyceraldehyde-3-phosphate Dehydrogenase GAPDH; EC 1.2.1.12	rat	?	MC		DO	3 days	Two times more mRNA under hypoxic incubation conditions	DNA	22
	Jaculus orientalis	?	M		NH	>1 month	3 isozymes present in hibernating and non-hibernating jerboas. GAPDH I had highest activity in euthermic animals, while GAPDH II had highest activity in hibernating animals. GAPDH III activity was a minor component in both groups. Total GAPDH activity was 3 − 4 × higher in euthermic jerboas	SDS-PAGE SPA WSB	23
	Thamnophis sirtalis parietalis	3	H	D T	P	3 weeks	Greater staining intensity in 12L:12D group than 0L:24D group	SGE	18
Glycerol-3-phosphate Dehydrogenase G3PDH; EC 1.1.1.8	*Cepaea nemoralis*	8	HP		AE			(D) PAGE	1
	Lepomis cyanellus	?	L		T			PAGE	17
Heat Shock Protein HSP	*Gillichthys mirabilis*	1 per treatment	B	—	T SC	18°C → 24, 28, 32 & 36°C 28°C → 28, 30, 32 & 36°C	Quantity of HSP90 increased as the heat shock temperature increased for both groups	PL LSC	24

TABLE 2. (*continued*)

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s) Fixed	Variable(s) Manipulated	Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
	Gillichthys mirabilis	3	B	—	SC	18°C (winter) 28°C (summer)	Summer fishes had higher levels of HSP90 than did winter fishes	WSB PAGE	24
	Gillichthys seta	1 per treatment	B	—	T SC	18°C → 24, 28, 32 & 36°C 28°C → 28, 30, 32 & 36°C	Quantity of HSP90 increased as the heat shock temperature increased for both groups	PL LSC	24
	Oncorhynchus mykiss	—	CC		T		High temperature or $NaAsO_2$ resulted in increases for 6 HSPs	SDS-PAGE PL mRNA	25
	Oncorhynchus tshawytscha	—	CC		T		HSPs synthesis (up) when T raised from 20 to 24 or 26°C; Actinomycin D prevented the increase	SDS-PAGE PL	26
	Oncorhynchus tshawytscha	?	CC		T Zn Cd		SP synthesis (up) when exposed to metals or high Ts; Actinomycin D prevented the increase	SDS-PAGE PL	26
Hemoglobin HB	*Ambloplites rupestris*	10 (cont.) 7 (exp.)	Bl	T pH, D	DO	3 ppm (exp.) 8–9 ppm (cont.)		PE	27
	Barbus holubi	?	Bl		SC		2 of 5 HBs exhibited minor changes at different seasons	DST PAGE CMH	28
	Barbus holubi	?	Bl		SC			DST PAGE	29
	Carassius auratus	12	Bl		T			PAGE, DST SPS	30
	Carassius auratus	12	Bl		T	5 & 30°C		AH, DST PAGE	31
	Carassius auratus	12	Bl		T			PAGE AH, DST	32
	Carassius auratus	8–14	Bl		T	15, 25 & 35°C 25° ± 0°C 15° → 25°C 25° → 35°C		CMH	33
	Channa punctatus	?, ♀	Bl		SC		Well-defined trend observed from Jan.–Dec.; higher in winter and early spring declining to lowest point in August	Haemophotometer	34
	Cyprinus carpio	?	Bl		SC		3 Hbs & total HB changed in abundance throughout different seasons	DST PAGE CMH	28
	Cyprinus carpio	6–8	Bl		T	5 & 30°C		DST PAGE	31
	Cyprinus carpio	?	Bl		SC			DST PAGE	29
	Cyprinus carpio x *Carassius auratus*	14–16	Bl		T	5 & 30°C		DST PAGE	31
	Catostomus commersoni	12	Bl		T	3, 10 & 30°C		DST PAGE	31

	Ictalurus punctatus	?	Bl	T, D pH	DO	3 week	Significantly higher HB at hypoxic conditions (@ 24, 48 & 72 hr hypoxic & 24 & 48 hr hypoxic and returned to normoxic	CMH	35
	Labeo capensis	?	Bl		SC			DST PAGE	29
	Labeo niloticus	?	Bl		SC			Measured Fe content	36
	Labeo umbratus	?	Bl		SC			DST PAGE	29
	Lates niloticus	?	Bl		SC			Measured Fe content	36
	Lepomis gibbosus	10	Bl		T	3, 10 & 20°C		DST PAGE	31
	Oncorhynchus mykiss	12	Bl		T	2, 10 & 18°C		PAGE, DST SPS	30
	Oncorhynchus mykiss	?	Bl		T DO P			DST CAGE	37
	Oncorhynchus mykiss	?	Bl		T	12–30 weeks		SPA? IEF	38
	Oncorhynchus mykiss	?	Bl	P, pH, D, OWP T not fixed, but same for all groups	DO	24 days	Normoxic and hypoxic differed for A1, A3, C3, C5, C6, C7 & C8	CAGE DST	39
	Pleuronectes americanus	10–12	Bl		SC		Total HB significantly lowered in winter vs summer fishes	AHM	40
	Salmo salar	?	Bl		SC			CMH	41
Invertase ---; EC 3.2.1.26	*Triticum aestivum vulgare*	?	LF	P	T	6 & 20°C	3 invertase fractions eluted with differences in intensity/activity between 6 & 20°C for Kharkov and Rescue varieties	Assay CHR	42
Isocitrate Dehydrogenase IDHP; EC 1.1.1.42	*Oncorhynchus mykiss*	?	L		T			CHR; DST IEF (H) SGE	43
	Salvelinus fontinalis	?	L		T			(H) SGE	44
Lactate Dehydrogenase LDH; EC 1.1.1.27	*Carassius auratus*	?	L		T			CAGE; DST PAGE; SPA	45
	Carassius auratus	5?	L	D	T	>2 months	Large quantitative differences in LDH 1, 2 & 3 between 18 & 28°C; LDH 1, 2 & 3 lower at 18°C	CAGE DST	46
	Cepaea nemoralis	8	HP		AE			(D) PAGE	47
	Discoglossus pictus pictus	? ♀	B L H		T		Decrease or increase in some LDHs between 8 and 18°C	PAGE DST	48
	Eptesicus fuscus	60(?)	M		NH		CA (15°C) → LDH 5 up CA (15°C) → LDH 2, 3, 4 down	CAGE DST, (V) SGE	49

TABLE 2. (*continued*)

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s) Fixed	Variable(s) Manipulated	Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
	Fundulus heteroclitus	?	M		T			(V) SGE	50
	Fundulus heteroclitus	?	L	S T	DO	14, 22, 28 & 35 days 1.5–2.5 ppm (exp.) 8–9 ppm (cont.)		SPA MPA (H) SGE	21
	Gillichthys mirabilis	?	L P	D	T	4–6 weeks	8°C fishes had much lower amount of all LDHs vs (28°C + 15L:9D) 28°C fishes (8°C + 11L:13D)	SGE DST	51
	Leuciscus idus	?	L		T		CA (10°C) → band 7 greater WA (20°C) → bands 5 & 8 greater	CAGE	52
	Lepomis cyanellus	?	M		T			PAGE SPA	17
	Myotis lucifugus	5 pooled	M L H		NH			SGE, DST (D) PAGE	53
	Rhodeus sericeus amarus	?	B		T		All five LDHs highest at 10°C, followed by 20°C, and lowest at 0°C	DST (D) PAGE	15
	Salvelinus fontinalis	?	M		T			SGE	54
	Salvelinus namaycush	?	M		T			SGE	54
	Semotilus atromaculatus	4	L		T			PAGE	55
	rat	?	MC		DO	3 day	Two times more mRNA under hypoxic incubation conditions	DNA	22
	Xenopus laevis	?	L, LC KC		T		Quantitative differences in some LDH between/among the experimental temperatures	(D) PAGE DST	56
α_2-Macroglobulin (protease inhibitor)	*Spermophilus tridecemlineatus*	?	L		SC		Increased activity and 2× mRNA in animals hibernating or nearing hibernation	Assay QSB	57
	Spermophilus richardsonii	?	L		SC		Increased activity and 2× mRNA in animals hibernating or nearly hibernating	Assay QSB	57
	Spermophilus columbianus	?	L		SC		Increased activity and 2× mRNA in animals hibernating or nearing hibernation	Assay QSB	57
Malate Dehydrogenase MDH; EC 1.1.1.37	*Fundulus heteroclitus*	?	L	S T	DO	14, 22, 28 & 35 days 1.5–2.5 ppm (exp.) 8–9 ppm (cont.)		SPA MPA (H) SGE	21

	Gillichthys mirabilis	9	M		T SC	6 weeks 10 & 30°C	Two MDH higher in 30°C and summer fish, and the third MDH higher in 10°C and winter fish	PAGE SPA	58
	Goniobasis cahawbensis	?	whole body		T	14 days	Increased at higher temperatures: 10°, 17° & 24°C	(H)SGE	13
	Lepomis cyanellus	?	B		T			PAGE	17
	Nicotiana tabacum	6?	LF	T P	P T			PAGE	59
Metallothioneins	*Oncorhynchus tshawytscha*	?	CC		Cd Zn		MT synthesis (up) when exposed to metals Actinomycin D prevented the increase	SDS-PAGE PL	26
Myosin Light Chain MLC	*Cyprinus carpio*	6–7	M	P D	T	6–12 weeks 8 & 20°C	Large quantitative differences in relative amounts of fast MLC ($MLC1_f$, $MLC2_f$) and slow MLC ($MLC1_s$, $MCL2_s$) between 8 ° and 20°C carp	SDS-PAGE	60
Peroxidase PER; EC 1.11.1.7	*Juniperus scopulorum*	4 R	F		SC			PAGE	61
	Nicotiana tabacum	6?	LF	T P	P T			PAGE	59
Phosphoglucomutase PGM; EC 5.4.2.2	*Lepomis cyanellus*	?	M		T			PAGE	17
Protein, General PROT	*Ambloplites rupestris*	9 (cont.) 7 (exp.)	Bl	T pH, D	DO	exp. = 3 ppm cont. = 8–9 ppm		PE	27
	Ambloplites rupestris	8 (cont. & exp.)	M	T pH, D	DO	exp. = 3 ppm cont. = 8–9 ppm		PE	27
	Brassica napus	?	Whole Seedling	P	T	0L:24D	Some proteins/polypeptides differed between 0 & 18°C	2DE	62
	Carassius auratus	10	M, L B		T	5 weeks	65 kDa polypeptide much higher in 30°C than 10°C fishes	SDS-PAGE IMC, 2DE	63
	Carrassius auratus	10	M, L B		T	5 weeks	28 & 200 kDa polypeptides much higher in 30°C than 10°C fish	SDS-PAGE	63
	Cyclonassa neritea	?	M HP		T			(G) PAGE	2
	Lepomis macrochirus	5 ♂	Bl S	P, D, AK T, pH	DO	cont. x = 8.3 ppm exp. x = 3.5 ppm		PE	64
	Micropterus salmoides	5 & 4 ♂	Bl S	P, D, AK T, pH	DO	cont. x = 8.1 ppm exp. x = 3.1 ppm		PE	64
	Notemigonus crysoleucas	many	Bl		SC		Several serum proteins varied during the year, some extreme. The variation differed between males and females.	PE	65
	Secale cereale	?	Crown MP?		T P			SDS-PAGE 2DE, IEF	66
	Spinacia oleracea	?	LF (nuclei)		T		Ca → 3 bands increased & 2 bands decreased	SDS-PAGE	67
	Trachemys scripta elegans	3	L	T	DO	3 weeks preacclimation 16 hr anoxia	28.6 & 79.9 kDa polypeptides increased in anoxic turtles	SDS-PAGE	68
	Triticum aestivum	?	Crown		T P	cont. → 7 day exp. → 40 day	16 proteins increased or decreased	SDS-PAGE 2DE, IEF	69

TABLE 2. (*continued*)

Enzyme	Organism	Number examined per treatment	Tissue	Variable(s) Fixed	Variable(s) Manipulated	Exposure time and conditions	Explanation or observed effects	Technique(s) used	Reference(s)
	Triticum aestivum	?	LF		T P	cont. → 7 day exp. → 40 day	7 proteins increased 12 proteins decreased	SDS-PAGE 2DE, IEF	69
	Triticum aestivum	?	Root		T P	cont. → 7 day exp. → 40 day	13 proteins increased 4 proteins decreased	SDS-PAGE 2DE, IEF	69
Pyruvate kinase PK; EC 2.7.1.40	rat	?	MC		DO	3 day	Two times more mRNA under hypoxic incubation conditions	DNA	22
Superoxide dismutase SOD; EC 1.15.1.11	*Crithidia luciliae thermophila*	—	whole/ cultures		T	25°C 33°C → 2 hr 37°C → 24 hr	Increase in total SOD activity and in quantity of the two main isozymes (pI 5.1 & 5.4) at 37°C vs 25°C groups	IEF SPA	7
Triose-phosphate isomerase TPI; EC 5.3.1.1	*Euglena gracilis*	—	Whole Organism		P M			IEF	16
	rat	?	MC		DO	3 day	Two times more mRNA under hypoxic incubation conditions	DNA	22

*Sixteen initially, 6 died, then 4 were added = 14.

Abbreviations: Number Examined: R, Resampled the same individuals under different conditions.

Tissues: B, Brain; BL, Blood; CC, Cell Cultures; E, Eye; ER, Ear; F, Foliage; H, Heart; HP, Hepatopancreas; I, Intestine; KC, Kidney Cell Line; LF, Leaf; L, Liver; LC, Liver Cell Line; M, Muscle; MA, Mantle Tissue; MC, Myogenic Cells; P, Prosoma; S, Serum; ST, Stem; Tf, Tube Feet; Wb, Whole Body.

Variables: AE, Active vs Estivating; AK, Alkalinity; D, Diet, DO, Dissolved Oxygen; NH, Normothermic vs Hibernating; P, Photoperiod; SC, Seasonal Collection; T, Temperature.

Techniques: AHM, Acid Hematin Method; CAGE, Cellulose Acetate Gel Electrophoresis; CHR, Chromatography; CMH, Cyanmethemoglobin Procedure; DNA, cDNA Probe; DST, Densitometry; FE, Foil Electrophoresis;IEF, Isoelectric Focusing; IMC, Immunochemistry; LSC, Liquid Scintillation Counting; MPA, Microbiuret Protein Assay; MW, Molecular Weight; PAGE, Polyacrylamide Gel Electrophoresis (D = Disc, G = Gradient Gels); PE, Paper Electrophoresis; PL, Protein Labelling; QSB, Quantitative Slot Blot; mRNA, *in vitro* RNA translation; SDS-PAGE, Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis; SGE, Starch Gel Electrophoresis (H = Horizontal, V = Vertical); SF9P, Serum Freezing Point; SMP, Serum Melting Point; SPA, Spectrophotometric Assay; TA, Titration Assay; TDE, Two-Dimensional Electrophresis; WSB, Western Blot.

Metals: Cd, Cadmium; Zn, Zinc.

References: 1, Gill, 1978b; 2, Trellu *et al.*, 1978; 3, Whitmore and Goldberg, 1972; 4, Yardley and Wild, 1991; 5, Borowsky, 1984b; 6, Braam and Davis, 1990; 7, Emtage and Bremmer, 1993; 8, Scandalios, 1990; 9, Sidell, 1977; 10, Wilson, 1973; 11, Karr *et al.*, 1985; 12, Marcus, 1977; 13, Pugh *et al.*, 1979; 14, Shaklee *et al.*, 1977 & James B. Shaklee, pers. comm.; 15, Hauss, 1975b; 16, Mo *et al.*,1973; 17, Wilson *et al.*, 1975; 18, Alesksiuk, 1976; 19, Hochachka and Clayton-Hochachka, 1973; 20, Chang *et al.*, 1979; 21, Greaney *et al.*, 1980; 22, Webster and Murphy, 1988; 23; Soukri *et al.*, 1995; 24, Dietz and Somero, 1992; 25, Kothary and Candido, 1982; 26, Heikkila *et al.*, 1982; 27, Bouck, 1972; 28, Fourie and van Vuren, 1976; 29, van Vuren and Hattingh, 1978; 30, Houston and Cyr, 1974; 31, Houston *et al.*, 1976; 32, Houston and Rupert, 1976; 33, Houston and Murad, 1992; 34, Mahajan and Dheer, 1979; 35, Scott and Rogers, 1981; 36, Mahdi, 1972; 37, Tun and Houston, 1986; 38, Weber *et al.*, 1976; 39, Marinsky *et al.*, 1990; 40, Umminger and Mahoney, 1972; 41, Hardig and Högland, 1984; 42, Roberts, 1975; 43, Moon and Hochachka, 1971b; 44, Moon and Hochachka, 1971a; 45, Yamawaki and Tsukuda, 1979; 46, Tsukuda, 1975; 47, Gill, 1978a; 48, DeCosta *et al.*, 1981; 49, Brush, 1968; 50, Bolaffi and Booke, 1974; 51, Somero, 1973; 52, Künnemann, 1973; 53, Moon, 1978; 54, Hochachka, 1967; 55, Kent and Hart, 1976; 56, Tsugawa, 1976; 57, Martin *et al.*, 1993; 58, Lin and Somero, 1995; 59, De Jong, 1973; 60, Langfeld *et al.*, 1991; 61, Kelley and Adams, 1977; 62, Meza-Basso *et al.*, 1986; 63, Kikuchi *et al.*, 1993; 64, Bouck and Ball, 1965; 65, Summerfelt, 1964; 66 Uemura and Yoshida, 1984; 67, Guy and Haskell, 1989; 68, Douglas *et al.*, 1994; 69, Perras and Sarhan, 1989.

dalios (394); the transcription and/or translation of many genes in plants is either directly or indirectly affected by light [also see (125,196)]. Additional cases of qualitative isozyme changes are given in Table 1. Marinsky *et al.* (300) studied the effects of hypoxia on HB in *O. mykiss* by first inducing anemia, then holding fishes under either normoxic or hypoxic conditions and sampling fishes at 14, 20, 22 and 24 days. Two HB isoforms exhibited qualitative variation between the two groups; HB isoforms C1 and A5 were present in normoxic but not hypoxic *O. mykiss*.

QUANTITATIVE ALTERATIONS OF PHENOTYPE

In addition to the qualitative changes mentioned above for *S. atromaculatus*, quantitative variation was also observed between the 5 and 20°C (8L:16D) groups (253). Moon and Hochachka (322) found the same phenomenon with IDHP isozymes in rainbow trout livers using SGE, IEF, SPA and CHR; the IDHP in *O. mykiss* did not exhibit a complete replacement of the isozymes but only quantitative changes. Moon and Hochachka (321) found splake trout (*S. namaycush* ♀ × *S. fontinalis* ♂) to undergo thermally induced shifts in hepatic IDHP, whereas brook trout (*S. fontinalis*) exhibited only quantitative changes and lake trout (*S. namaycush*) showed no such variation; therefore, the observed variation in splake likely resulted from the expression of *S. fontinalis* genes in the splake. The quantitative changes noted for IDHP in brook and splake trout may have constituted a qualitative change involving isoloci and therefore may have been masked. *Idhp*-1 and *Idhp*-2 exist as isoloci (*Idhp*-1,2) in *O. mykiss* liver (275). Distinction of isoloci and null alleles often relies on gene dosage effects (472); therefore, quantitative variation due to environmental factors would be problematic in making such distinctions and also may change the phenotype enough to suggest the involvement of isoloci or null alleles when in fact this may not be the case.

Elevation of the incubation temperature of *Oncorhynchus tshawytscha* embryo cells from 20°C to 24–26°C resulted in greater quantitative expression of HSP based on labeling with L-[35^S]methionine and SDS-PAGE (193). Cultures at 20°C treated with Actinomycin D for 30 min before heat shock did not exhibit the response of untreated heat shocked cultures; thus, suggesting control at the transcriptional level. Continuous exposure of the fish cells to 28°C resulted in constant elevated expression of the HSP (192). Similar results were found for *O. mykiss* cultured cells (267). Whitmore and Goldberg (499) used PAGE, DST and SPA to examine ALP in thermally acclimated *S. fontinalis*, *S. namaycush* and splake. Quantitative changes were found for *S. fontinalis* between the 4°C group and the 10°C and 15°C temperature groups using PAGE. Three mechanisms that may have caused the ALP variation were suggested: alteration of isozyme synthesis, conformational changes and aggregation in gel or alteration of membrane lipids that in turn affect the activity and/or structure of membrane-bound enzymes such as ALP (499). Studies of SUDH by Hazel (181,182) and ATPase by Smith and Kemp (423) indicate that the third mechanism above could very well be the cause of the ALP variation in *S. fontinalis*. Kelley and Adams (251) analyzed foliage of four *Juniperus scopulorum* collected monthly for 1 year and found quantitative alterations in PER using PAGE. Several calmodulin and calmodulin-related genes were induced 10- to 100-fold (mRNA) in *Arabidopsis* sp. due to water spray, subirrigation, wind, touch, wounding or darkness. Maximum mRNA levels were rapidly attained (10–30 min) and decreased greatly by 1–2 hr (45). Environmental factors can affect the hematology of *Morone saxatilis*; HB was significantly lower in saltwater-acclimated as compared with freshwater-acclimated individuals (84). Plasma protein levels were lower in cold-acclimated saltwater fishes than in warm-acclimated saltwater fishes. Courtois (84) controlled diet, photoperiod, dissolved O_2 (>6 ppm) and varied salinity and temperature combinations.

Wilson *et al.* (512) acclimated *Lepomis cyanellus* to 5 and 25°C and discovered quantitative variations for muscle LDH, FBALD and PGM, liver G3PDH and brain MDH as determined by staining intensity on starch gels. Of the quantitative variations observed by Wilson *et al.* (512), the liver G3PDH variation was most pronounced as seen in their Fig. 4 C. SPA of muscle LDH activity was in accord with the differences in staining intensity between 5 and 25°C fishes based on DST scans of LDH on polyacrylamide gels (512). Enzymes investigated in *L. cyanellus* did not exhibit any qualitative thermally induced isozymes changes; however, there were rather large quantitative variations in some of the EST [(406); J.B. Shaklee, personal communication]. EST-2, EST-4 and EST-6 from eye extracts were present in greater quantity in 5°C fishes than in 20°C fishes. Fishes held at 5°C also possessed increased amounts of liver EST-1 and EST-2. In garter snakes (*Thamnophis sirtalis parietalis*) acclimated to 4°C and either 0L:24D or 12L:12D photoperiods, heart FBALD and GAPDH were stained much less intensely after SGE in the 0L:24D group (n = 3) than the 12L:12D group (n = 3); no photoperiod effect was observed for heart LDH, MDH or sIDHP (7). *Euglena gracilis* exhibited quantitative shifts in FBALD and TPI between light and dark conditions (319). When grown under light, chloroplastic FBALD and TPI predominate, whereas cytoplasmic FBALD and TPI predominate under dark conditions. Chloramphenicol prevented the increase of chloroplastic FBALD and TPI under photic conditions, thus supporting *de novo* synthesis of these enzymes under light conditions. Mo *et al.* (319) also cited other cases of environmentally regulated gene expression for ALP, GAPDH, MDH and FBALD. Quantitative variation was noted for EST, CAP and PER in *Citrus* spp. and for EST and PER in *Poncirus trifoliata* between plants held under either a long or short photoperiod (489).

Bouck and Ball (43) found quantitative variations in se-

rum proteins of *Micropterus salmoides* (two of six fractions) and *Lepomis macrochirus* (one of five fractions) between normoxic and hypoxic (3.1–3.5 ppm) conditions. Three of six plasma protein fractions and HB varied quantitatively between *Ambloplites rupestris* that experienced diurnal hypoxia and those that did not (42). Marinsky *et al.* (300) studied the effects of hypoxia on HB in *O. mykiss* by first inducing anemia and then holding fishes under either normoxic or hypoxic conditions and sampling fishes at 14, 20, 22 and 24 days. Significant quantitative differences were observed for seven HB isoforms between normoxic and hypoxic fishes based on CAGE and DST analyses. Goldfish acclimated to normoxic and hypoxic conditions at 15 and 30°C exhibited significant differences in activity of some enzymes in red muscle, white muscle and liver. Greater differences were noted between normoxic and hypoxic groups at 30°C than 15°C (452). Shaklee *et al.* (406) tested for the effects of oxygen concentration and temperature on several enzymes in *L. cyanellus* and concluded that oxygen concentration had no significant effect on isozyme changes. Additional cases of altered quantitative expression are given in Table 2.

Few acclimation studies examined both changes in enzyme activity and gel staining intensity. A positive linear relationship was found for *L. cyanellus* muscle LDH activity (SPA) vs gel staining intensity (DST) (512). Differences in activities are not always accompanied by changes in gel staining intensity. A number of enzymes in various tissues of *L. cyanellus* displayed changes in activity using SPA, whereas gel patterns remained unchanged (406). Although the change in staining intensity noted by Wilson *et al.* (512) for muscle LDH was not observed by Shaklee *et al.* (406), both studies reported higher specific activity for 25°C fishes. Sun (445) stated: "In fact, no matter what kind of staining is used, the protein concentration is not always proportional to its staining intensity. The relationship is linear up to a certain concentration, but as the concentration of protein becomes too dense, incomplete staining may result."

ACCLIMATION EXPERIMENTS IN WHICH NO QUALITATIVE OR QUANTITATIVE CHANGES WERE OBSERVED

Studies of other species have shown no changes in isozymes over wide temperature ranges. Somero (430) tested for thermally induced qualitative isozyme changes in *Ameiurus nebulosus*, *Gillichthys mirabilis*, *Gibbonsia elegans*, *G. metzi* and *G. montereyensis* and observed no changes. Similarly, Giles and Vanstone (148) concluded that coho salmon (*Oncorhynchus kisutch*) HB were not altered by the variety of temperatures, salinities and levels of dissolved O_2 used in their research. Powers (357) acclimated *Catostomus* spp. to 2, 5, 10, 15, 20, 25 and 30°C for 2 months and examined HB by SGE. No qualitative changes were mentioned. HB content per erythrocyte was also unchanged, but erythrocyte number decreased at lower temperatures (357). Smit and Hattingh (420) also found no significant change in HB in three fish species held under hypoxic conditions. Johnson (239) noted that holding *Anoplarchus purpurescens* at temperatures between 1 and 24°C for up to 2 months did not alter gel patterns of LDH allozymes. *Gillichthys mirabilis* muscle LDH was not qualitatively affected (on SGE) by acclimation to 15 vs 25°C (488). Similarly, Greaney *et al.* (165) electrophoretically examined liver and white skeletal muscle of *Fundulus heteroclitus* acclimated to hypoxic or normoxic conditions and found no qualitative changes for any of the three enzymes (LDH, MDH and GPI). Marangos and Constantinides (298) mentioned that they observed no variation in winter flounder, *Pleuronectes americanus*, GAPDH "isozymes" in fish collected at different seasons. Also, no qualitative changes were detected in *Taricha granulosa* between 5 and 20°C groups for LDH, MDH and IDHP (130); however, only a 2-week acclimation period was tested. Rome *et al.* (385) reviewed thermal acclimation in amphibians; they found no evidence of qualitative thermally induced isozyme changes, although several studies they cited had reported changes in relative amounts of LDH A and LDH B. If the literature examined by Rome *et al.* (385) constitutes the majority of acclimation/enzyme studies on amphibians, then only a few enzymes have been investigated for change in amphibians and caution should be used in inferring that such changes are not possible. Pinder *et al.* (346) also indicated that no instances of qualitative thermally mediated isozyme/allozyme changes had been reported in the literature. However, seasonal changes in enzyme activities are common (in anurans) and relate mainly to the seasonal alterations in feeding (summer) vs starving (winter) (346). Acclimation of crayfishes, *Cambarus bartoni*, to 9°C (9L:15D) or 25°C (12L:12D) for 3 weeks did not reveal differences in electrophoretic patterns of LDH or G3PDH (356). Somero and Hochachka (437) remarked that "Acclimation experiments which have failed to find changes, e.g. in isozyme patterns, may have failed to induce the changes the organisms are capable of making, if the laboratory experimental regime involved variation in temperature but not in photoperiod." Certainly, this line of reasoning can be extended to other variables such as salinity, pH or dissolved O_2 [see (165)].

SUBUNIT REARRANGEMENTS/ENZYME STABILITY

The concept of on-off regulation of isozymes, which would account for observed qualitative and quantitative variation, is very intriguing; however, an interesting study by Houston and Rupert (222) provides us with yet another possible explanation for at least some of the observations of previous studies. *Carassius auratus* acclimated to 3 and 23°C possess two and three HB, respectively. Goldfish held under diurnally cycling temperatures in the range of ≈3°C to ≈20°C possessed two HB when sampled at ≈3°C or three HB when

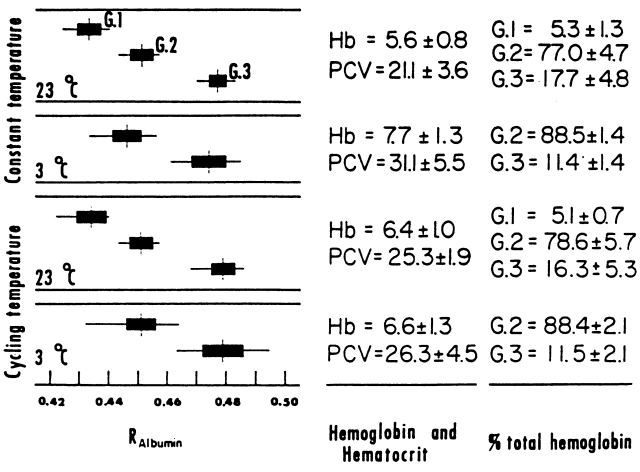

FIG. 4. Hemoglobin isoforms and HB quantity from 3 and 23°C acclimated *Carassius auratus* (From Houston and Rupert [222], with kind permission from Arthur H. Houston, Brock University, St. Catharines, Ontario, Canada.)

sampled at ≈23°C, which is consistent with data from the constant temperature groups. A third treatment involved the abrupt exposure of previously acclimated fishes (at either 3 or 23°C for 3 weeks) to the alternate temperature, after which samples were collected hourly for 12 hours. Changes from a two HB pattern into a three HB pattern and *vice versa* required only 3 hours! The same changes could also be produced *in vitro*! Also, as the warm-inducible third HB disappeared, the other two HB were quantitatively increased and *vice versa* (Fig. 4). The results of Houston and Rupert indicate that rearrangements of subunits may be responsible for the HB variation due to temperature in the goldfish and not a *de novo* synthesis/repression (on-off) system. Regardless of the cause, the fact that such changes occur in the products visualized electrophoretically means that interpretation may be misled. Contrary to the reports of multiple HB in goldfish (219,222), other investigators reported only one HB in goldfish [Braunitzer and Rodewald in (331), (400,476)]. Perhaps such differences exist between geographically distant populations of the species, especially because a number of different ploidy levels have been found in goldfish or different species (possibly undescribed) have been involved (449) (Table 3). *Cyprinus carpio* in South Africa had two HB during the summer (July–November), whereas three HB were present in winter (November–June) (142). Hashimoto and Matsuura (177) detected one or two HB in *C. carpio*, whereas Summerfelt (444) reported only one. Perhaps a similar phenomenon that occurs in goldfish HB also occurs in carp HB.

From these data it is tempting to assign some adaptive significance to the changes. It certainly seems plausible; however, another possibility is that the disappearance of an isozyme or isoform is simply due to structural instability (temperature- and/or pH-mediated changes in weak bonding) imposed by the new conditions to which the organisms have been exposed. The reaggregation of the subunits into the other isozymes (isomorphs) that were already present may compensate for the loss. The differential stability of isozymes is well known [e.g., LDH in (403,502)]; however, the temperatures routinely used are far above the biologically meaningful range, except in Shaklee (403).

Instability has been shown for the LDH heterotetramers, A_1B_3 and A_3B_1, of *Alosa pseudoharengus* (403). All tissues examined expressed non-binomial LDH patterns, but *in vitro* molecular hybridization of LDH A_4 and B_4 produced the expected binomial distribution. The A_1B_3 was more unstable than A_3B_1, and examination of activity vs time for A_1B_3 in several buffers revealed that activity is lost faster at more alkaline pHs. Such occurrences could certainly produce quantitative and possibly qualitative differences; however, no specific cases have been found that demonstrated this. "Restricted" expression of LDH heterotetramers has also been found in the lizard family Xantusiidae (417). *In vitro* molecular hybridization was not used to determine if the heterotetramers could be formed. Many other examples of restricted expression have also been published [see references in (417)].

Phosphofructokinase in *Spermophilus* (= *Citellus*) *beecheyi* is a tetramer in its active form and dissociates to inactive dimers at low temperature/low pH (174); the inactive dimers recombine and activity is restored by 1 hr after the temperature or pH rises to the appropriate level. Rabbit muscle PFK exhibits a similar pH- and temperature-related dissociation (157). This phenomenon may be important to mammals, but what about in lower vertebrates? *Cyprinus carpio* PFK is also very pH sensitive, and the activity of carp PFK could drop as much as 60% of maximum with a 0.1 pH unit change under physiological substrate concentrations (145). PEPCX in plants can also dissociate into dimers upon cold treatment of extracts; the dimers retain some activity, and therefore one may misinterpret results concerning numbers of isozymes or polymorphism [(256) and references therein]. At any rate, consider the analysis of mammalian PFK by electrophoresis. Under the conditions used for SGE, the PFK may migrate as dimers, tetramers or possibly both, and dimers may recombine to active tetramers during staining, thus giving an additional "false" band. Support for the above concerning PFK may be seen in the example of *Escherichia coli* CS. *E. coli* CS exists as three forms in equilibrium: monomer (inactive), dimer (active) and tetramer (inactive). However, three bands appear after electrophoresis and even after reelectrophoresis of any one of the original bands (97). The PFK of *O. mykiss*, which supposedly exists as different isozymes after long-term temperature acclimation, may be inducible in the short term if inactive dimers for both isozymes were present at all times. Temperature and buffer concentration have been shown to influence the monomer-dimer equilibrium of IDHP isozymes in the fish, *Fundulus heteroclitus* (160). Armstrong (13) mentioned a temperature-sensitive form of tyrosine 3-

TABLE 3. Fishes, amphibians, reptiles and hybrids considered to be polyploid (including unisexual forms)

Species	Ploidy	Reference
Sarcopterygii		
Lepidosireniformes		
Protopteridae		
Protopterus dolloi	4n	Vervoort, 1980
Actinopterygii		
Acipenseriformes		
Acipenseridae		
Acipenser baeri	8n	
Acipenser brevirostrum	12n	Blacklidge and Bidwell, 1993
Acipenser fulvescens	8n	Blacklidge and Bidewell, 1993
Acipenser güldenstädti	8n	Birstein and Vasilév, 1987
Acipenser medirostris (North American)	8n	Blacklidge and Bidwell, 1993
Acipenser medirostris (Asian)	16n?	Birstein *et al.*, 1993
Acipenser nacarii	8n	
Acipenser nudiventris	4n	
Acipenser oxyrhynchus desotoi	4n	
Acipenser ruthenus	4n	Ráb, 1986; Birstein and Vasilév, 1987
Acipenser schrencki	8n	
Acipenser sinensis	8n	Yu *et al.*, 1987
Acipenser stellatus	4n	Birstein and Vasilév, 1987
Acipenser sturio	4n	
Acipenser transmontanus	8n	Blacklidge and Bidwell, 1993
Huso dauricus	4n	
Huso huso	4n	Birstein and Vasilév, 1987
Pseudoscaphirhynchus kaufmanni	4n	Birstein *et al.*, 1993
Scaphirhynchus platorynchus	4n	
Polyodontidae		
Polyodon spathula	4n	Dingerkus and Howell, 1976
Salmoniformes		
Salmonidae (entire family)	4n	Allendorf and Thorgaard, 1984; Allendorf *et al.*, 1975
Cypriniformes		
Cyprinidae (only part of family)		
Opsariichthys uncirostris bidens	3n?	Yu *et al.*, 1987
Zacco platypus	3n?	Yu *et al.*, 1987
Diptychus sp.	4n	
Diptychus dipogon (2n = 446)	17n or 18n?	Yu and Yu, 1990
Schizothorax davidi	4n?	
Schizothorax taliensis	6n	
Schizothorax grahami	6n	
Schizothorax prenanti	6n	
Schizothorax niger	4n	
Schizothorax sp.	6n	
Acrossocheilus sumatranus	4n	
Barbodes (Spinibarbus) caldwelli	4n	
Barbodes (Spinibarbus) d. denticulatus	4n	
Barbodes (Spinibarbus) sinensis	4n	
Percocypris p. pingi	4n	
Sinocyclocheilus g. grahami	4n	
Sinocyclocheilus grahami tingi	4n	
Sinocyclocheilus maculatus	4n	
Tor khudree	4n	
Tor mosal mahanadicus	4n	
Tor putitora	4n	
Tor tor	4n	
Aulopyge hugeli	4n	
Barbus barbus	4n	Engel *et al.*, 1971
Barbus bocagei	4n	
Barbus brachycephalus	4n	
Barbus comiza	4n	
Barbus m. meridionalis	4n	
Barbus meridionalis petenyi	4n	

TABLE 3. (*continued*)

Species	Ploidy	Reference
Barbus microcephalus	4n	
Barbus occidentalis	4n	Agnèse *et al.*, 1990
Barbus petitjeani	4n	
Barbus plebejus	4n	
Barbus sacratus	4n	Agnèse *et al.*, 1990
Barbus sclateri	4n	
Barbus steindachneri	4n	
Barbus tauricus cubanicus	4n	
Barbus wurtzi	4n	Agnèse *et al.*, 1990
Rutilus alburnoides (some "populations")	3n	Collares-Pereira, 1989
Iberocypris palacciosi	2n, 3n, 4n	
Carassioides cantonesis	4n	
Carassius a. auratus	4n	
Carassius auratus gibelio	3n, 4n, 6n	
Carassius auratus langsdorfi	3n, 4n, 6n, 8n	
Carassius carassius	4n	Engel *et al.*, 1971; Sola *et al.*, 1981 (2n in Collares-Pereira, 1989)
Cyprinus c. carpio	4n	Engel *et al.*, 1971
Cyprinus carpio chilia	4n	
Cyprinus carpio rubrofuscus	4n	
Cyprinus longipectoralis	4n	
Cyprinus megalophthalmus	4n	
Cyprinus pellegrini barbatus	4n	
Cyprinus (Mesocyprinus) micristius fuxianensis	4n	
Procypris rabaudi	4n	
Perlampus atpar	3n?	Tripathi and Sharma, 1987
Phoxinus eos-neogaeus (F_1 hybrid)†	2n–3n	
Phoxinus eos-neogaeus x *eos*†	3n	
Phoxinus eos-neogaeus x *neogaeus*†	3n	
Catostomidae (entire family)	4n	Ferris, 1984; Uyeno and Smith, 1972; Tsoi *et al.*, 1989
Cobitidae (only part of family)		
Botia hymenophysa	4n	Ferris and Whitt, 1977b
Botia macracanthus	4n	Ferris and Whitt, 1977b
Botia modesta	4n	Ferris and Whitt, 1977b
Botia pulchra	4n	Yu *et al.*, 1987
Botia robusta	4n	Yu *et al.*, 1987
Misgurnus fossilis	4n	Raicu and Taisescu, 1972
Misgurnus anguillicaudatus (some populations)	4n	Yu *et al.*, 1987
(Loaches)	4n	Hitotsumachi *et al.*, 1969
Cobitis sinensis (some populations)	4n	Yu *et al.*, 1987
Cobitis biwae (some populations)	4n	Kobayasi, 1976; Ueno and Ojima, 1976; Hitotsumachi *et al.*, 1969
Cobitis t. taenia (some populations)	4n	Ueno and Ojima, 1976
Cobitis taenia striata (some populations)	4n	Ueno and Ojima, 1976
Cobitis taenia x unknown sp. (2)	3n	Vasilév *et al.*, 1989
Cobitis taenia (2) x unknown sp. (2)	4n	Vasilév *et al.*, 1989
Cobitis taenia x unknown sp. (2) x *granoei*	4n	Vasilév *et al.*, 1989
Siluriformes		
Callichthyidae (only part of family)		
Corydoras aeneus	5n or 6n?	Scheel *et al.*, 1972
Corydoras aeneus	4n	Dunham *et al.*, 1980
Corydoras agassizi	4n	Scheel *et al.*, 1972
Corydoras julii	4n	Scheel *et al.*, 1972; Dunham *et al.*, 1980
Corydoras metae	4n	Scheel *et al.*, 1972
Corydoras cf. *osteocardus*	3n or 4n	Scheel *et al.*, 1972
Cranoglanididae		
Cranoglanis sinensis	3n?	Yu *et al.*, 1987
Atheriniformes		
Poeciliidae (these "biotypes" or "hybrid species" are all gynogenetic)		
Poecilia mexicana-2 latipinna	3n	Schultz, 1980

TABLE 3. (*continued*)

Species	Ploidy	Reference
Poecilia 2 mexicana-latipinna	3n	Schultz, 1980
Poecilia mexicana-latipinna-''limantouri''	3n	
Poeciliopsis 2 monacha-lucida	3n	Schultz, 1980; Vrijenhoek, 1984
Poeciliopsis monacha-2 lucida	3n	Schultz, 1980; Vrijenhoek, 1984
Poeciliopsis-monacha-lucida-viriosa	3n	Schultz, 1980; Vrijenhoek, 1984
Perciformes		
Percidae		
Lucioperca sandra (Finland population(s))	4n	
Amphibia		
Anura		
Pipidae		
Silurana epitropicalis	4n	
Xenopus amieti	8n	
Xenopus andrei	8n	
Xenopus borealis	4n	
Xenopus boumbaensis	8n	
Xenopus clivii	4n	
Xenopus fraseri	4n	
Xenopus gilli	4n	
Xenopus laevis	4n	
Xenopus muelleri	4n	
Xenopus ruwenzoriensis	12n	
Xenopus vestitus	8n	
Xenopus wittei	8n	
Leptodactylidae		
Ceratophrys dorsata	8n	
Ceratophyrs ornata	8n	
Odontophrynus americanus	4n	
Pleurodema bibroni	4n	
Pleurodema kriegi	4n	
Hylidae		
Hyla versicolor	4n	
Phyllomedusa burmeisteri	4n	
Bufonidae		
Bufo danatensis	4n	
Bufo viridis	4n	
Bufo sp. D	4n	
Ranidae		
Dicroglossus occipitalis	4n	
Pxyicephalus delalandii	4n	
Rana esculenta	4n	
Rana ridibunda x *lessonae* (2)	3n	
Rana ridibunda (2) x *lessonae*	3n	
Caudata		
Ambystomatidae		
Ambystoma laterale-(2) *jeffersonianum* (*A. platineum*)	3n	
Ambystoma laterale-(3) *jeffersonianum*	4n	
Ambystoma (2) *laterale-jeffersonianum* (*A. tremblayi*)	3n	
Ambystoma (3) *laterale-jeffersonianum*	4n	
Ambystoma (2) *laterale-*(2) *jeffersonianum*	4n	
Ambystoma (4) *laterale-jeffersonium*	5n	
Ambystoma (2) *laterale-texanum*	3n	
Ambystoma laterale-(2) *texanum*	3n	
Ambystoma (3) *laterale-texanum*	4n	
Ambystoma laterale-(3) *texanum*	4n	
Ambystoma (2) *laterale-*(2) *texanum*	4n	
Ambystoma laterale-jeffersonianum-texanum	3n	
Ambystoma laterale-texanum-tigrinum (*A. nothogenes*)	3n	
Ambystoma laterale-(2) *texanum-tigrinum*	4n	
Ambystoma laterale-(2) *jeffersonianum-texanum*	4n	
Ambystoma laterale-(2) *jeffersonianum-tigrinum*	4n	

TABLE 3. (*continued*)

Species	Ploidy	Reference
Reptilia		
Testudinata		
Chelidae		
Platemys platycephala	2n–3n, 3n, 4n	Bickham *et al.*, 1993
Squamata		
Gekkonidae		
Gehyra variegata	3n	
Hemidactylus garnotti	3n	
Hemidactylus vietnamensis	3n	
Heteronotia binoei	3n	
Agamidae		
Leiolepis triploida	3n	
Teiidae		
Cnemidophorus exsanguis	3n	
Cnemidophorus flagellicaudus	3n	
Cnemidophorus lemniscatus	2n, 3n	
Cnemidophorus opatae	3n	
Cnemidophorus sonorae	3n	
Cnemidophorus tesselatus	3n	
Cnemidophorus uniparens	3n	
Cnemidophorus velox	3n	

The following references were the primary sources used for compiling Table 3: Buth *et al.*, 1991; Buth, 1983, 1984a; Blacklidge and Bidwell, 1993; Birstein *et al.*, 1993; Sola *et al.*, 1981; Vasilév, 1980; Yu *et al.*, 1987; Bogart, 1980; Cannatella and de Sá, 1993; Licht and Bogart, 1987; Lowcock *et al.*, 1987; Vrijenhoek *et al.*, 1989; Goddard *et al.*, 1989; Collares-Pereira, 1989; Ráb and Collares-Pereira, 1995.

†Possible offspring of $F_1 \times$ *Phoxinus* spp.; reproduction of the F_1 hybrid is a variation of gynogenesis.

monooxygenase (EC 1.14.16.2) that is responsible for the pigmentation patterns of Siamese cats. In the extremities, which experience lower temperatures, the enzyme is active and melanin is produced, whereas in other parts of the body where the temperature is higher, the enzyme is relatively inactive. Acromelanic albinism is the term applied to the type of albinism found in Siamese cats, as well as rabbits, guinea pigs and mice (382).

The experimental conditions under which enzymes are assayed have a profound effect on the observed activity. Kleczkowski and Edwards (256) discussed conflicting literature reports on PEPCX physical and catalytic properties and suggested that cold storage and other conditions of sample preparation and assay be considered possible causes for observed differences. The conditions often used, such as constant pH at a number of temperatures, are biologically unrealistic (214). Also, the kinetic properties of enzymes determined under such conditions may greatly exaggerate the effects of temperature on the *in vivo* enzymatic activity [see Fig. 11–12 in (214)] and this may partly explain the distinctive differences in K_m of the cold and warm AChE isozymes in rainbow trout. Detection of significant differences between acclimation groups may be dependent on assay temperature; activity of HK (white muscle) and PFK (red muscle) were significantly different between 5 and 18°C *Coregonus clupeaformis* when measured at 20°C but not at 5°C (constant pH) (34). Shikata *et al.* (408) also observed activity differences for several enzymes (e.g., PK, PFK and FBP) at different assay temperatures.

In vivo, pH changes accompany changes in temperature (65,357,358,432) (Fig. 5); this relationship conserves the K_m value of an enzyme by conserving the charge state of histidine imidazole groups that are important for enzyme function. This process is termed alphastat regulation and allows enzyme function to continue at near optimal rates (65,432). The pH change associated with temperature change may be accomplished by a bicarbonate exchange system (194,495) or by another mechanism (194); most eukaryotic organisms apparently have several pH regulatory systems, although their interaction is not well understood (293). Internal pH changes induced by temperature have been shown to alter enzyme activity; Hazel and Prosser (189) discussed this scheme as a control mechanism for thermal compensation. Cases of temperature-dependent pH optima have also been demonstrated (see 189). Carp muscle FBP exhibits temperature-dependent pH optima with lower pH optima at higher temperatures (388), agreeing well with the alphastat regulation hypothesis. Membrane lipid fluidity at low vs high temperatures is also affected somewhat by temperature-dependent pH regulation (188).

Somero (431,432) discussed the importance of duplicating *in vitro* the *in situ* microenvironment in which a protein normally functions as this could markedly alter the structure and/or function of the enzyme. It seems likely that changes within the cellular microenvironment *in vivo* during thermal acclimation or due to some other stimulus will potentially alter the structure of enzymes. Somero (433) concludes: "It follows that, if one wishes to examine proteins *in vitro* under experimental conditions that closely mimic

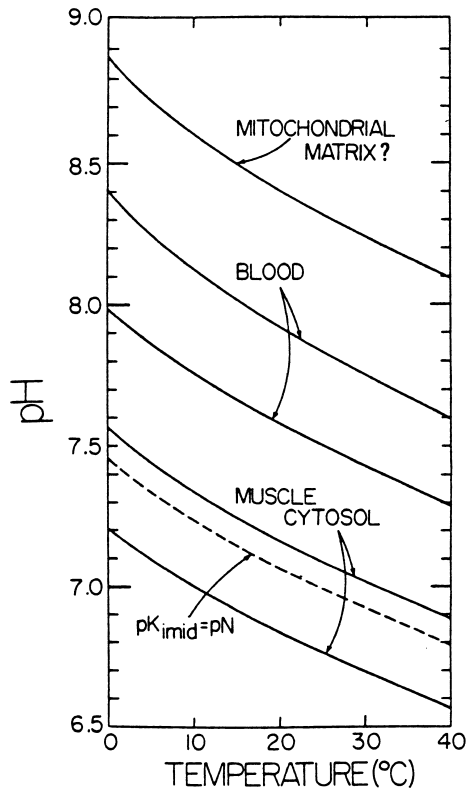

FIG. 5. pH changes with temperature changes *in vivo*. (From Somero, G.N. pH-temperature interactions on proteins: Principles of optimal pH and buffer system design. Mar. Biol. Lett. 2:163–178;1981, with kind permission from Elsevier/North Holland Biomedical Press.)

the *in situ* microenvironment of the proteins, one must duplicate the osmolyte composition of the cellular fluids in question." Realistic assay conditions should be used, particularly with regard to temperature; at least one of the assay temperatures should be the temperature to which the organism had been acclimated [e.g., (17,44,218)], and organisms should have been exposed to similar temperatures (533). Similarly, Behrisch *et al.* (27) suggested a refined experimental methodology that included attempting to match the physiological state of an organism. An interesting approach was used by Greenfield and Greenfield (166); fish muscle was homogenized in freshwater teleost physiological saline solution rather than a more typical solvent such as distilled water or buffer.

DURATION OF EXPERIMENTAL ACCLIMATION PERIOD

Acclimation times used in past research have varied from several weeks to many months (see Tables 1 and 2). Three to 4 weeks appears to have been the most common acclimation period. Some studies have acclimated animals for long periods and taken subsamples at various intervals. But what is the appropriate acclimation period that should be used? For example, Duman and DeVries (118) showed that under laboratory conditions the times of production and loss of antifreeze glycoproteins varied among the species tested. The acclimation period should be sufficient to induce a change that is capable of manifesting itself; however, one does not know *a priori* if a change will occur or how long it may take.

Three to 4 weeks has proven to be a satisfactory time to induce a number of qualitative enzyme alterations, but could these changes have occurred in 2 weeks or even a few days? Changes in goldfish HB that were detected after several weeks of acclimation also were inducible within a matter of a few hr *in vitro* (222). Qualitative changes in EST patterns of sea urchins did not occur in 7 days and required approximately 14 days (299). Conversely, could changes be overlooked if the period of acclimation is too prolonged? Greaney *et al.* (165) acclimated *Fundulus heteroclitus* to hypoxic conditions for 35 days, sampling five to seven fishes at 14, 22, 28 and 35 days. Activities of hepatic LDH, MDH and GPI were significantly increased over normoxic controls at days 14, 22, and 28, but the activities had returned to control levels by day 35. Increased levels of liver protein were also found at 14, 22, 28 and 35 days. Greaney *et al.* (165) pointed out that had they not sampled fishes at intervals before day 35, the observed increases in activity would have been missed altogether. Sidell *et al.* (414) observed oscillations in CYTOX, SUDH and CAT activity in *C. auratus* undergoing acclimation from 15 to 5°C and 15 to 25°C. A relatively steady state (narrow range) of enzyme activity was reached between 3 and 4 weeks. Massaro and Booke (303) also subsampled *F. heteroclitus* in their study of LDH; 20 control and 20 treatment fishes were sampled every 30 days for a 210-day period. Kent and Hart (253) sampled four fishes at 14 days and four fishes at 21 days from each acclimation group of eight fishes. Two to 3 weeks were required for the changes observed by Kent and Hart (253). There is no single ideal or standard acclimation period *per se*; however, from past research it would appear that several elements should be included in the experimental acclimation period. A minimum period of 3–4 weeks seems wise, whereas the maximum has no definite limits. Even within the minimum time span, samples should be taken at shorter intervals. Acclimation experiments designed after Houston and Rupert (222), using long-term and short-term (*in vivo*) treatments as well as *in vitro* treatments, should provide a better understanding of the types of mechanisms operating for specific proteins in different species exposed to various environmental conditions.

ENVIRONMENTALLY INDUCED CHANGES IN ENZYME ACTIVITY

A number of studies have noted altered enzyme activities with changes in acclimation temperature; these include ChE in *Fundulus heteroclitus* brain (21), HK in *Mytilus edulis* mantle tissue (284), ALAT in *Misgurnus fossilis* muscle

(316), choline acetyltransferase in *Carassius auratus* brain (191), CYTOX in *C. auratus* muscle (144), PFK in *C. auratus* muscle (145), CAP in *O. mykiss* plasma (44), MFO in F. heteroclitus liver (440), MFO in *O. mykiss* and *Leuciscus rutilus* liver (110), eight enzymes in red muscle, seven in white muscle and five in liver of *C. auratus* (452), CYTOX in *Sebastes miniatus* and *S. auriculatus* muscle (513), AChE in *Lepomis macrochirus* brain (215), AChE in *C. auratus* brain (180), FBALD and PGDH in *C. carassius* gills (123), MDH in *Drosophila melanogaster* (4), MDH in *Cyprinus carpio* muscle (270), SUDH and G6PDH in *Blennius pholis* liver (67), CS in *Lepomis cyanellus* and *Micropterus dolomieu* red muscle (264), CYTOX and CS in *Coregonus clupeaformis* white muscle (34), PDH in *O. mykiss* liver (484), LDH in *C. auratus* red and white muscle (462) and several enzymes in various tissues of *Esox niger* (255) and *Lepomis cyanellus* (406). Seasonal changes in G6PDH activity have been found in *Mytilus edulis* (283) and in LDH, MDH and AAT in brain, gills and liver and ALAT in liver and muscle of *Ictalurus punctatus* (307). Significant differences in SOD and CAT were found in some cases for *S. namaycush*, *Catostomus commersoni* and *Margariscus margarita* taken from the same lake but at different times of year. Similarly, changes were also found for GPX, but only for *C. commersoni* and *M. margarita* (340). Major LDH activity differences were seen between groups of northern anchovy, *Engraulis mordax*, exposed to different acclimation conditions (435). Bhaskar and Govindappa (29) acclimated *Oreochromis mossambicus* [(*Tilapia mossambica* in (381)] to pHs of 5.0, 9.0 and 7.0 (control) for 15 days and examined enzyme activities from the three groups (averaged activities from eight individuals). Enzyme activities from pH 5.0 fishes that differed from the control group were GPase (−53%), FBALD (−34%), G6PDH (+57%), LDH (+40%), SUDH (+33%) and MDH (+65%). Changes between the control group and the pH 9.0 group were as follows: GPase (+20%), FBALD (+30%), G6PDH (−30%), LDH (−11%), SUDH (+67%) and MDH (+9%). Activity of CAP in *Mytilus edulis* varied with acclimation to different salinities with increased CAP activity at higher salinities and *vice versa* (261). Variation in enzyme activity due to body size differences have also been described [LDH, PK and CS in (435); AChE in (533); MFO in (2,235)]. However, in the abovementioned studies the enzymes were not quantified so there is no direct evidence for quantitative (or qualitative) alterations in enzyme expression. Regarding CAP in *Mytilus edulis*, Koehn *et al.* (262) did quantify CAP from mussels acclimated to different salinities. Rocket immunoelectrophoresis indicated that enzyme quantity changes did not accompany the salinity-mediated activity changes.

ENVIRONMENTALLY INDUCED ALTERATIONS OR GENETIC POLYMORPHISMS?

The reports of qualitative thermally induced isozyme changes have been suggested by several researchers to be due to genetic polymorphism. Hazel (187) stated ". . . many early studies failed to distinguish between genetic polymorphism and isozyme induction. . . ." Shaklee *et al.* (406) also pointed out the possibility of misconstruing a polymorphism for a qualitative isozyme change. In their (406) initial sample of four acclimated fishes (two at 5°C, two at 25°C), there appeared to be a thermally induced isozyme change in PGM; however, when additional fishes from each acclimation temperature were examined, this relationship was shown to be a result of polymorphism only (the four original fishes were homozygotes, f/f & s/s, whereas additional fishes included some heterozygotes, f/s) and not thermally induced alterations. Shaklee *et al.* (406) suggested using a minimum of five individuals at each acclimation temperature as a safeguard against the above situation. Wilson *et al.* (514) also pointed out the importance of adequate sample sizes when investigating enzyme variation.

It would then appear that little doubt could be cast upon the results of Baldwin and Hochachka (17) as they pooled brains from between 30 and 80 *O. mykiss* for each of the three acclimation temperatures. Kent and Hart (253) used four individuals at each acclimation temperature/photoperiod, whereas Massaro and Booke (303) used 20 control and 20 treatment *F. heteroclitus* every 30 days for 210 days. Marcus (299), Kelley and Adams (251), Oxford (337), McGovern and Tracy (310,311), Baker *et al.* (16) and Gill (149,150) all used samples taken from the same individual; the samples were taken from the individuals after they had been exposed to differing conditions than when the initial samples were collected. Therefore, the failure to distinguish between genetic polymorphism and qualitative isozyme change in early studies appears to have been unlikely based on the number of individuals sampled and/or the repeated sampling of a single individual, at least in the specific cases cited above. That is not to say that the results of the abovementioned studies are conclusive as to the cause of the observed phenomena. As mentioned previously, possible explanations involve post-translational modifications and contamination of samples with other tissues, but these are not genetic polymorphisms either. It is important to consider that in studies in which specimens are not acclimated "multiple variants" may also be construed as genetic polymorphisms when they may actually be the products of different loci, post-translational modifications or allelic with repression of one allele occurring (see below).

Wilson *et al.* (514) discussed potential sources of error in acclimation studies (and these would apply to any study as well). Muscle is a widely analyzed tissues in electrophoretic studies and is typically present in most fishes as two basic types: red (slow/oxidative) and white (fast/glycolytic) fibers (37,244). These two types of skeletal muscle are distinct as far as their role in locomotion and may also vary with respect to isozyme expression (132,244). Smit *et al.* (421) demonstrated a distinct difference between LDH isozyme patterns of red and white muscle from *C. auratus*; the red muscle pattern was quite similar to the complement of iso-

zymes found in the heart. The relative contribution of red and white muscle in a homogenate should be considered as a source of variation, as these seem to possess unique expression of LDH isozymes (quantitatively; ratio of A to B subunits) (511,514) and also vary in amount of enzyme activities [e.g., LDH, PK and CS in (435)] Kleckner and Sidell (255) analyzed enzyme activity in red and white muscle separately in their acclimation study of *Esox niger*. Sidell (412) also examined enzyme activities separately for red and white muscle. Three muscle types can be distinguished in some species of fish; the carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*) and several marine teleosts from Australian waters contain slow (red), intermediate (pink) and fast (white) muscle types (242,244,326,412). Each type of muscle within the carp contained a distinct isoform of myosin heavy chain (242). Discrete examination of each of the three muscle types may also show differences with respect to particular isozymes. The relative proportions of red and white muscle have been shown to change with different acclimation temperatures (more red muscle at colder temperatures) in *Lepomis cyanellus*, *Micropterus dolomieu*, *Carassius auratus* and *Morone saxatilis* (243,245,264,412,421). Several investigators have also found differences in numbers of mitochondria between groups of temperature-acclimated fish (67,122). Therefore, quantitative changes in enzymes may be partially due to changes in size or number of cells and/or organelles rather than simply an increase or decrease per cell/organelle (413). Recent reviews of fish muscle temperature adaptation were provided by Johnston (241) and Goldspink (158). Several enzymes, PFK, HK, FBALD and GAPDH, occur as both membrane-bound and free enzymes within at least some tissues, and some quantitative or activity differences could simply be due to changes in the free:bound ratio and not the amount of enzyme actually present (474).

Sidell (411) discussed the possible importance of enzyme half-lives as a component of the ability to respond and acclimate to rapidly changing conditions experienced by some poikilothermic organisms. Sidell (411) pointed out that at least for cytochrome *c* mammalian enzyme half-lives were much longer than those in poikilotherms. Another possible cause for observed quantitative enzyme alterations may be changes in the rates of synthesis or degradation (392,413,437). If the rate of synthesis remains unchanged, but the degradation rate increases, the total enzyme content will decline. Conversely, if the degradation rate declines and the rate of synthesis remains constant, the enzyme level would increase. Cytochrome *c* increased quantitatively in cold-acclimated vs warm-acclimated green sunfish; both synthesis and degradation rates declined during cold acclimation. However, the degradation rate declined more in comparison with the rate of synthesis, resulting in higher levels of cytochrome *c* in cold-acclimated fishes (411).

Several investigators have postulated the basic mechanisms by which poikilotherms adjust to temperature changes and maintain relatively temperature-independent metabolic rates (27,213,222,323,427,437,499). These mechanisms include

1. "On-off" synthesis or translation of different stenothermal or stenotolerant isozymes or allozymes that have a functional advantage under certain conditions: two genes and alternative splicing of one split gene;
2. Production of a single eurythermal or eurytolerant enzyme that functions well at "normal" biological conditions;
3. Changes in the concentrations of specific isozymes within a set of isozymes, depending on the conditions of exposure and which isozyme functions best under those conditions;
4. Changes in the lipid moiety of lipoproteins and membranes allows for activity to change in order to maintain proper function under different conditions;
5. Conformational changes in a single enzyme that produces kinetically distinct forms suited to particular conditions;
6. Production of a single enzyme that is functionally altered by intracellular changes in activators or inhibitors;
7. Rearrangements of existing polypeptides to produce more of the isozyme(s) that function(s) well under the new conditions (or loss of a certain isozyme may be due to structural instability of that particular aggregation and have nothing to do with adapting to new conditions).

Any of the above, individually or in concert, could be responsible for the maintenance of biological function and for the variation observed in acclimation studies. Levels of gene products can also be controlled at a number of other levels between transcription and the final product (100,392,393), including regulatory gene control. Somero (429,430) referred to 1 above as the "Multiple Variant" strategy of adaptation and 2 as the "Eurythermal" strategy. An organism displaying a multiple variant isozyme or allozyme system (two or more stenothermal enzymes) would increase its fitness by producing only one enzyme form (the most efficient) at a particular temperature. The multiple variant isozymes of Somero (429,430) can refer to enzyme groups that respond to temperature changes, other environmental variables or physiological changes that produce intra- or extracellular alterations that in turn produce an enzyme shift. Further discussion concerning the "costs and benefits" of each of the suggested strategies of biochemical adaptation is not pursued here; however, for a more in-depth treatment, refer to Somero (433), Somero and Hochachka (437), Somero and Low (438) and Hochachka and Somero (214).

TEMPERATURE-DEPENDENT CONFORMATIONAL CHANGES IN ENZYMES

Baldwin and Reed (18) found evidence that two distinct forms of sMEP were exhibited in *O. mykiss* acclimated to temperatures of 5 and 20°C. Electrophoretic analysis did

not reveal any differences between warm- and cold-acclimated fishes; however, kinetic studies indicated that two distinct enzymes were present in the two groups. Whether these two forms of sMEP were encoded by different genes or were simply derivatives or interconversions of the same product was undetermined. Allendorf and Thorgaard (9) reported MEP to exist as isoloci in *O. mykiss* (identical alleles in common between the two loci). Although the electrophoretic mobilities were identical even after acclimation to different temperatures and acknowledging that some products of isoloci comigrate during electrophoresis, the K_m differences noted by Baldwin and Reed (18) may indicate that the expression of the two isoloci have diverged temporally (i.e., regulated seasonally with temperature changes). The kinetic differences between warm and cold sMEP were observed under both 5 and 20°C assays. Amino acid sequence data for the two sMEP proteins would be most informative concerning the mechanism of isozyme formation. Somero (426) reported two distinct forms of PK from leg muscle of nonacclimated Alaskan king crab (*Paralithodes camtschatica*) based on kinetic differences at different assay temperatures. However, as with the sMEP examined by Baldwin and Reed (18), electrophoresis failed to distinguish two forms; IEF was also unable to reveal any differences among the samples. Somero (426) presented evidence that a temperature-dependent interconversion may be operating. A similar phenomenon may have occurred in LDH from *Misgurnus fossilis* (339). Interconversion of PK has also been noted in rat adipose tissue (348) and other mammalian PK [references in (426)]. Powers and Powers (358) found the E_a of *Fundulus heteroclitus* LDH-B_a B_a to differ between temperatures above and below ≈12.4°C. The E_a was ≈11.7 kcal/mol above 12.4°C and ≈50.7 kcal/mol below 12.4°C. The other LDH B tetramers, B_aB_b and B_bB_b, had E_a of ≈11.1 kcal/mol that did not change with temperature. Powers and Powers (358) suggested a temperature-dependent conformational change may be responsible for the change in LDH B_aB_a E_a. Evidently, this change was also not electrophoretically detectable. Wolfe and Gray (517,518) acclimated *O. mykiss* to either 5 or 15°C for 3 weeks and then examined GAPDH in the two groups. A single band of GAPDH activity with identical mobility was found in both groups. However, the enzyme from cold-acclimated trout differed from the warm enzyme in K_m, E_a, ability to bind NAD and circular dicroism spectra (kinetic parameters determined at constant pH). A conformational difference between the warm and cold GAPDH was postulated (518). The warm and cold isozymes of GAPDH were also immunologically distinct, which would support their distinctiveness either due to a stable conformational change or different primary structures (369). Because GAPDH is a tetramer, homozygotes would exhibit one band of activity and heterozygotes five bands (1:4:6:4:1) on gel electrophoresis. It is interesting that all the *O. mykiss* GAPDH always exhibited one band of activity (369, 518), indicating that only homozygous trout were examined. How would temperature acclimation affect the GAPDH in heterozygous trout? *O. mykiss* possess two GAPDH loci that have diverged to the point that common alleles are not shared between loci (9); therefore, one would expect more than one band of activity on a gel even for a homozygote at both loci. Marangos and Constantinides (298) examined GAPDH from muscle and liver of *Pleuronectes americanus* and found three isozymes in muscle and five isozymes in liver. Based on their analyses, Marangos and Constantinides (298) concluded that the multiple isozymes were due to conformational differences arising within a tetramer composed of identical subunits.

A thermally induced change in K_m has been documented in *Zea mays* leaf PEPCX; a change in conformation or oligomerization was postulated as the probable cause for the altered kinetics between PEPCX assayed at cold and warm temperatures (256). Lebherz (276) discussed several accounts of conformational isozymes and useful methods for investigating this phenomenon. Lebherz (276) also mentioned that most cases of "conformational isozymes" have provided little evidence that a conformational change has actually taken place. Somero and Hochachka (437) provided a detailed discussion of conformational isozymes; two basic models were outlined to explain how a protein may undergo a conformational change. The "metastable" model involves a protein that undergoes a series of folding events after its synthesis. If a change in temperature occurs, instead of newly synthesized proteins assuming the final conformation (after its last fold), it will assume the conformation resulting at an earlier stage in the series of folds due to an energy barrier "blocking" the subsequent fold or folds. Therefore, over time the enzyme conformation present before the temperature change will become less abundant, and the new conformation will become more abundant due to normal turnover, until only the new conformation is present. The multistable model allows for an individual protein already present to undergo a change in conformation due to a change in temperature, therefore assuming a presumably more stable conformation. Basically, the metastable model would apply to changes *in vivo* only, whereas the multistable model could apply to changes *in vivo* as well as *in vitro*. The instantaneous isozymes of Somero (426) would be formed according to the multistable model. Such conformational changes may allow a single protein to function over a wide temperature range (437). Liver mitochondrial F_1-ATPase (EC 3.6.1.3) has also been shown to exhibit temperature-dependent conversions based on analyses of catalytic properties and several spectroscopic measurements (425).

Actually, changes in protein conformation are a common occurrence due to binding of substrates and modulators (209,248), and some of these changes can be extreme (297,373). Many weak bonds involved in protein structure are temperature sensitive (209,213,214). Conformational changes could arise due to restructuring imposed by changes in weak bonds (Van der Waals interactions, hydrogen bonds, ionic bonds, hydrophobic interactions). Thermally

induced changes in weak bonds could not only change the completed polypeptide conformation but may also affect its construction or even be involved in the control of gene expression. If a repressor protein or molecule has its bonding altered due to temperature alterations in weak bonds, gene transcription could be initiated, halted or changed to varying degrees as a direct result of temperature (158,213). Therefore, some of the cases in Table 1 may be directly controlled by temperature. The question is, will changes in any of these temperature-sensitive weak bonds be sufficient to induce a conformational and/or charge change which will be detectable by electrophoresis? At least in some cases (426,518), the change was not sufficient to produce detectable electrophoretic differences. From the published studies discussed above, one might conclude that the use of K_m differences to determine distinct isozymes could very well be unreliable when comparing samples that differed in acclimation or were assayed under varying conditions of temperature, pH, and so on. Somero and Hochachka (437) warned against assuming that kinetically distinct isozymes from organisms acclimated to different conditions represent the products of different alleles or loci. Enzymes may undergo cyclic conformational changes resulting in oscillations in activity with frequencies of several minutes (409). Bell and Bell (28) discussed conformational changes and experimental methodologies for detecting conformational changes. Also, Curtil *et al.* (96) described an interesting method for the observation of temperature-induced conformational changes.

THE POSSIBLE RELATION OF POLYPLOIDY TO ENVIRONMENTALLY INDUCED ENZYMES

Utter *et al.* (472:32) stated that "The salmonids have undergone the most intensive electrophoretic examination of any group of fishes, and the complexities of their electrophoretic patterns have often been confusing. . . ." Most electrophoretic investigations of salmonid systematics or population genetics have not taken into account the findings of Baldwin and Hochachka (17) and other acclimation studies; therefore, thermally induced enzyme alterations may be an underlying cause of the difficulty in interpreting zymograms in salmonid research, in addition to the presence of multiple genetic loci in the Salmonidae, which are the result of past gene duplications (9). Derived fishes possess more loci and more tissue-specific expression of isozymes (503–505); therefore, would these fish also tend to have more temperature-specific expression as well? The duplicate loci in tetraploids would potentially allow divergence to isozymes expressed at different temperatures; this idea has also been previously suggested by Somero (429–431), Hochachka and Somero (214) and Goldspink (158). If the above hypothesis is true, one may expect to find similar phenomena in the catostomids, some cyprinids, cobitids, acipenseriforms and others that have also undergone polyploidization events, resulting in various ploidy levels (9,127,134,136–138,332,364,475,481) (Table 3). Polyploidy may have arisen several times in acipenserids based on average chromosome numbers that center around 60, 120 or 240 chromosomes (481) and DNA content (33). The tetraploid state of the catostomids probably originated in the Asian catostomids before their geographical radiation (459). Catostomid chromosomes segregate disomically, whereas some salmonid chromosomes still segregate tetrasomically, and this is likely due to the allotetraploid origin of the catostomids vs the autotetraploid origin of the salmonids (9,133,134). Therefore, if thermal-specific isozymes are present in salmonids, they may be present in the genomes of other tetraploid fishes. Other poikilothermic vertebrate groups have tetraploid members as well (Table 3). Many plants are polyploids (64,329), and some species have several ploidy levels (64). A number of insects are also known polyploids (286).

Triploids may also be prone to expressing inducible isozymes. Occasional instances of triploidy may arise in a species that is functionally diploid; spontaneous triploids have been found in *O. mykiss* (454), *S. fontinalis* (Allen and Stanley in 454), *Pimephales promelas* (155), *Hesperoleucus symmetricus* (156), *Ginglymostoma cirratum* (252), *Ameiurus nebulosus* (81), *Acipenser fulvescens* and *A. oxyrhynchus desotoi* (33), *Tinca tinca* (141), *Noemacheilus barbatulus* (78) and *Agkistrodon piscivorus leucostoma* (455). In a full sib family of 11 individuals, Thorgaard and Gall (454) found 3 female and 3 male triploids. The females had female characteristics but poorly developed gonads, whereas the males had normal male characteristics and partially developed testes. Without karyological analysis, the triploids would have likely been assumed normal diploid individuals. *Tinca tinca* triploids were able to be separated from diploids with 96% accuracy by pelvic fin characteristics (141). The probable reason for the production of triploids is the pairing of a diploid egg with a normal sperm and the occurrence of triploids may not be uncommon in some cases (141,454). *Rana pipiens* females have produced diploid ova at a frequency as high as 35% (375). Davisson (102) noted that "A low frequency of what appeared to be polyploid cells (usually 4*n*) was observed in the meiotic tissues of both female muskellunge and male pickerel." Triploidy also results from a diploid-tetraploid cross (147,231,290). Triploidy and tetraploidy can be induced by heat shock, cold shock, pressure shock, hypoxia and chemical treatments [(19), review in (231,233)]. Tetraploidy in some cases may have arisen not as a single doubling event but rather through the production of triploids (ova $2n$ + sperm $1n$), which subsequently became tetraploids ($3n$ gamete + $1n$ gamete), possibly within only several generations.

An electrophoretic study of spontaneous triploid fishes would be quite interesting, because on top of the already polyploid background of some species is imposed an extra contribution from one parent (likely the female). Quantita-

tive differences in some enzyme expression would likely result, because a double dose of some genes would be present (98). Indeed, Dawley *et al.* (104) discovered exactly this in their electrophoretic and cytogenetic analyses of hybrid sunfish and backcross progeny. Asymmetrical staining patterns were observed in the triploid backcrosses, which contained a double dose of one species' genome (backcrosses were produced from female hybrids and males of either species; hence the double contribution from one of the species). Vrijenhoek (485) noted distinct asymmetric staining of ADH isozymes in the gynogenetic triploids, *Poeciliopsis* 2 *monacha-lucida* and *P. monacha-2 lucida*, which possess a double dose of one species' genome. Gene dosage effects have also been demonstrated in mosaic (2*n*-3*n*) and triploids of *Phoxinus eos-neogaeus* and *P. eos-neogaeus* x *P.* spp. (105,154). DeMaggio and Lambrukos (108) noted both increased and decreased quantitative expression (using DST and SPA) of PER with increased ploidy in ferns. Gene dosage effects have also been noted for *Zea mays* CAT-1 and CAT-2 enzyme levels (458).

Double gene doses have clearly been shown to affect the quantitative nature of isozyme expression; therefore, the occasional occurrence of triploids in an electrophoretic study may produce some interesting results that may not be so easily explained. The interpretation and explanation of zymograms from a triploid or mosaic (2*n*-3*n* or 3*n*-4*n*) that possess different ploidy levels in various tissues would surely be difficult in the absence of cytological data. With the discovery of several unisexual "species," or species complexes, consisting of several ploidy levels, one can imagine the variability in electrophoretic patterns may be confusing without any cytological data (103). In fact, one may consider the variation to be due to regulatory genes and classify them as quantitative "variants" (see classifications in 404). Confusion may also arise if a triploid (either from hybridization or spontaneous) is heterozygous for a monomeric protein from an unreduced ova and has a third unique allele contributed paternally; in this case a three banded staining pattern (1:1:1) would result for a monomeric protein. Wilson *et al.* (512:204) stated "It is clear that future work must explore such issues as the effect of polyploidy upon isozyme changes during temperature acclimation." The search for isozyme replacements or multiple variants should not be limited to polyploids only, because qualitative changes have been noted in the diploid fish species, *S. atromaculatus* (253), although the nature of the changes remains unknown.

GENE SILENCING OR RESTRICTED EXPRESSION?

The apparent lack of duplicated genes may not be a case of "losing" one of the pair through functional diploidization/gene silencing (92,136) but may be due to divergence limiting expression to subcellular conditions, ontogenetic stages and/or environmental conditions (134). Certainly, it should not be taken to mean that all cases of gene silencing or null alleles are due to restricted expression but only that some may be masked in this manner. The "loss" of duplicate gene expression may consist of four possibilities: disfunction, temporal shift in expression (usually manifested as a short-term appearance early in development) [e.g., (71,405,503)], spatial shift in expression (tissue-specific) (503,504) and divergence and assumption of a new function (138,278). Another possibility similar to the second choice above is expression restricted to a particular physiological condition which is thermally mediated, photoperiod-mediated, and so on, any of which may be either directly or indirectly controlled.

Just as an entire locus may be "masked" (repressed) or "unmasked" (derepressed) under certain conditions so may a single allele at a locus. A basic assumption in biochemical genetic studies is the codominant expression of alleles encoding polypeptide(s) of an enzyme (329). Null alleles may cause a deviation from the expected codominant expression (329). It seems at least plausible in some cases that a null allele could be either a repressed allele or a recessive allele paired with a dominant allele. Variations of environmental conditions may initiate expression of a null allele. Most expression is probably codominant, however, which allows one to distinguish between heterozygotes and homozygotes (377).

There are studies demonstrating both asynchronous and synchronous expression of maternally and paternally derived alleles in F_1 hybrid offspring (72,99,143,195,203, 257,258,335,341,345,397,503,519). This demonstrates that different alleles at a single locus can be controlled (expressed) independently of one another. Therefore, it would seem reasonable to postulate that qualitative isozyme changes need not necessarily involve two loci but instead two alleles at only one locus (213,429,430). Danzmann *et al.* (99) suggested a *cis*-acting genetic element was responsible for the regulation of ontogenetic expression of alleles at *Idhp-3* in *O. mykiss*. This same type of control may manifest itself in the control of allozymes that are thermally (or other variable) induced. Refer to Chandlee and Scandalios (73) for a review of regulatory genes in *Mus musculus*, *Zea mays*, *Drosophila melanogaster* and *O. mykiss*.

DIETARY-INDUCED ALTERATIONS (QUANTITATIVE AND QUALITATIVE)

A well-known example of enzyme induction is the lac enzymes of *E. coli*. The three enzymes (including β-galactosidase, EC 3.2.1.23) are normally present at low levels in the absence of lactose, but their synthesis is greatly increased after the addition of lactose to the culture medium. A regulatory or repressor protein blocks transcription in the absence of the inducer, allolactose. This example has been greatly simplified and greater detail can be found in Stryer

(442). Induction of aGLU was described for *Mycoplasma laidlawii*; the addition of maltose to the growth medium resulted in ten times the aGLU activity compared with glucose medium and was due to a quantitative increase in aGLU by increased synthesis (418). Ogihara (334) demonstrated dietary-induced quantitative differences in liver LDH patterns from both human and rat (*Rattus rattus*), and Purdom *et al.* (362) found variation in rat liver enzyme activities among six diets differing in carbohydrate composition. Diets high in fructose produced significantly higher activities of G6PDH, GPI and TPI. Oxford (337,338) also found diet to influence some enzymes in the snails *Cepaea nemoralis* and *C. hortensis*. It is possible that proteolytic degradation was responsible for some of Oxford's results because the hepatopancreas was the tissue used; therefore, the altered diet may have induced a new form of digestive enzyme or the bacterial gut flora may have been involved as postulated by Oxford (338). Activities of several proteolytic enzymes were much higher in liver tissue of fasting vs feeding rabbits, and one result was the transformation of native FBP into a secondary FBP with unique catalytic properties and electrophoretic mobility (351–355). Cold- or winter-acclimation produced the same result on rabbit liver FBP (see above). Activity differences were found for *Drosophila pseudoobscura* AMY between groups reared on different media (525), and ADH isozymes were qualitatively affected in six *Drosophila* spp. between diets with and without 2% 2-propanol (5). Yardley and Wild (526) studied the effects of a number of diets on AMY expression in *Gambusia holbrooki*. Quantitative differences in AMY resulted from dietary differences; in some cases activity differences were evident, but increased activity was not always associated with increased quantity of AMY. Many other cases of dietary-induced activity changes were cited by Yardley and Wild (526). Borowsky (41) tested dietary effects on expression of AMY in *Gammarus palustris* and discovered that an amylose or glycogen diet or low temperature (4°C vs 22°C) increased the expression of AMY-X (later called AMY I^c). Kinetic studies performed on the cold and warm isozymes (AMY I^c and AMY I^w, respectively) revealed little difference between the two isozymes; therefore, the differential expression does not appear to be adaptive (168). Temperature regulation may have evolved as a secondary or backup mechanism related to seasonal changes in available food. Other possible explanations for the two isozymes are differences in sialic acid content (139) or proteolytic modification (see 351–354). Fasted *G. palustris* did not show an increase in AMY I^c, however (41). Electrophoretic mobility of hepatic α-amylase differed between fasted vs well-fed rats and was shown to be the result of glycogen attachment to the enzyme in well-fed rats (447).

Research on proteins in blood sera of deer (*Odocoileus* spp.) uncovered both quantitative and qualitative changes in phenotypes as expressed on starch gels (477). Quantitative variations in some proteins were noted for *O. hemionus* captured during different seasons for different sexes and between fawns and adults. Qualitative differences occurred between wild captured and captive reared *O. hemionus* (originating from the same population). Possible causes for the variation were considered, and diet was thought to be the most likely cause due to the fact that the diets differ drastically between wild and captive animals. Also, differences were noted in the cloudiness of sera samples. In *O. virginianus*, increased mobility in fraction +7 and a quantitative increase in fraction +1 was related to greater cloudiness of the sera samples, which van Tets and Cowan (477) thought may be due to higher blood lipid levels (which could be ultimately due to dietary differences). The cause(s) of the variation was not specifically determined. van Tets and Cowan (477) pointed out that their study did not use enough animals to adequately rule out causes such as genetic polymorphism. A potential cause not pointed out by van Tets and Cowan (477) was the blood collection and handling differences between captive and wild animals. However, one can only speculate as to the true cause(s) of the variation.

Significantly higher activities of hepatic and renal CAT, SOD and GPX were the result in rats fed a restricted diet as opposed to those fed *ad libitum*. Liver mRNA levels paralleled the enzyme activity differences between the two groups of rats, thereby indicating altered rates of transcription at the *Sod*, *Cat* and *Gpx* loci (370). Rao *et al.* (370) indicated that gene expression can be altered by dietary restriction both transcriptionally and translationally. Dietary differences (including fasting) were shown to influence activity of hepatic HK in *Rattus norvegicus* and *Mus musculus* with some of the observed changes being quite extreme (471). Fishes experiencing hypoxia may decrease or cease feeding (42); therefore, electrophoretic variation may be due to the combined effects of hypoxia and dietary differences.

Salvelinus fontinalis starved for 2 months had lower hepatic G6PDH activity than fed trout, but activity returned to normal levels after refeeding (521). Comparisons of starved (6 weeks) and fed trout G6PDH and PGDH at both 5 and 10°C indicated little difference between fed and starved trout at 5°C, whereas 10°C significant differences were observed for G6PDH and PGDH activities. Liver weight also varied greatly between fed and starved trout at 10°C. GDH activity was significantly higher in starved trout at both temperatures (521). Whiting and Wiggs (496) found dietary differences and starvation to influence the activity of hepatic TAT in yearling *S. fontinalis*. Cowey *et al.* (88) fed *O. mykiss* high carbohydrate (HC) and high protein (HP) diets. Hepatic PK activity was significantly higher in the HC trout, whereas PEPCK and FBP activities were significantly higher in HP trout. HK activity was barely altered between HC and HP trout (88). Starvation of *Anoplopoma fimbria* resulted in lower CYTOX activities (443). Qualitative differences in HB of *Salmo salar* were possibly

due to the salinity or diet experienced by experimental groups of fishes (260). Starved *S. salar* possessed higher activities in liver of HK, GPase and PFK; under the same conditions in *Gadus morhua*, HK activity decreased (446). Therefore, as with other factors, different species may respond differently to the same treatment. Note that altered activities do not necessarily indicate quantitative changes. Whitmore and Goldberg (499) considered the possibility that diet had been at least partially responsible for the nongenetic variation found in trout ALP (350). Webster and Murphy (493) reviewed known regulators of enzyme levels based on changes in mRNA levels for GK, FBALD, TPI, ENO, PK, PFK, GAPDH, LDH, PEPCK and TAT. Hormones, glucose and cAMP were common regulators of levels of the enzymes listed above [refer to (493) for further information]. Injection of JH into *Hyalophora gloveri* pupae (in which JH is absent) induces the expression of two or three EST (carboxylesterases) not present in controls (500,501). Puromycin, cycloheximide, or actinomycin D prevented the appearance of the additional EST isozymes; therefore, *de novo* synthesis of the EST was supported. Alternative explanations given by Whitmore *et al.* (500,501) were that previously synthesized and bound EST were released or the EST isozymes were present as proenzymes until administration of JH. In either case, due to the inhibitory effects of puromycin, cycloheximide and actinomycin D, it appears that *de novo* synthesis of some sort is required, perhaps synthesis of an activator enzyme (500,501).

SEX-RELATED CHANGES (QUANTITATIVE AND QUALITATIVE)

Differences in enzyme activities may exist between males and females of a species. Zimmerman *et al.* (532) noted differences in activities of several enzymes from male and female *Gambusia holbrooki*. The differences in activity levels from site to site were also consistent between the sexes. Hepatic TAT activities differ between the sexes of *S. fontinalis* (497). Sex hormones may be responsible for some differences between the sexes (497). Oki *et al.* (336) found BChE activity to be greatly increased in pregnant mice as indicated by staining intensity after SGE, and Schmidt and Schmidt (396) found plasma BChE activity 5.5 times higher in adult female vs male rats. All rats had been held under strictly controlled environmental conditions (396). Gravid females of both *O. mykiss* and *O. keta* possessed an additional serum fraction capable of binding iron (175). The female-specific protein migrated close to TF on CAGE and could likely be TF with differences in the number of bound Fe^{3+} atoms; Zaragoza *et al.* (531) found pregnant rabbits possessed more of the unsaturated forms of TF than did males. The addition of iron to extracts allows for total saturation of all TF and minimizes nongenetic variation due to differences in the number of bound Fe^{3+} atoms. Hara (175) also cited other cases of female-specific serum proteins in fishes, amphibians and birds. Male and female golden shiners (*Notemigonus crysoleucas*) exhibited quantitative seasonal variation in serum protein patterns, and some proteins differed greatly in quantity between the sexes (444). Sun (445) indicated that increased levels of TF often accompanies pregnancy in humans, and Scandalios (391) discussed pregnancy-related changes in serum CAP gel patterns. Three additional CAP bands were observed in women at term; the additional bands appeared sequentially during the course of the pregnancy. The CAP gel phenotype of women returned to the "normal" pattern (one band) within a week after parturition (391). Both freshwater and anadromous *Gasterosteus aculeatus* from British Columbia were found to be sexually dimorphic for mIDHP, and it was suggested that mIDHP in males may undergo some form of PTM (515). Morgan *et al.* (325) found the distribution of 14 serum proteins from *Morone saxatilis* related to the sex as well as age and day of collection. Baker *et al.* (16) noted age-related qualitative and quantitative changes in plasma EST patterns. Transferrins in voles, *Clethrionomys glareolus*, exhibited marked quantitative (and possibly qualitative) variation over time, and the variation was related to the reproductive cycle of females (169). Gulyaeva and Olenev (169) also noted TF phenotypic changes in a preliminary analysis of *Microtus*. Whenever possible, the sex of specimens should be determined and tested for any correlation with particular phenotypes or levels of enzyme activity.

DETECTING CRYPTIC GENETIC VARIATION BY VARYING ENVIRONMENTAL CONDITIONS

The use of variably expressed isozymes or allozymes in systematic studies may provide valuable information just as do ontogenetically expressed isozymes (405), apparent regulatory variations (251) and the developmental patterns in hybrid fishes (341). Intraspecific differences in tolerance to certain pollutants may be due in part to genetic differences at loci coding for monooxygenases. Such differences are known among various strains of mice (124). Chagnon and Guttman (70) also suggested the utility that their metal tests may have in uncovering cryptic alleles. This technique could be very useful in systematic studies [see p. 1145 in (70) for a discussion of techniques for revealing cryptic variation]. Just as the metal tests may uncover cryptic alleles so may temperature acclimation experiments; temperature acclimation could potentially reveal alleles/loci which would not have been expressed; in other words, it is another way to uncover cryptic variation. Massaro and Booke (304) considered the above idea when they discovered a mutant LDH A allele in *Fundulus heteroclitus*. They used variations in temperature and photoperiod in an attempt to "enhance" the occurrence of the mutant allele. When these tests resulted in no new cases of the allele, they concluded that, in fact, the case they had uncovered was simply a mutant allele and not a case of environmentally induced isozyme

alteration. Duman and DeVries (118) found a genetic difference between northern and southern populations of *Anoplarchus purpurescens* in the ability to produce AFGP. The northern population (Alaskan) synthesized AFGP when acclimated to an appropriate temperature, whereas the southern (Californian) population failed to produce the AFGP under identical conditions. Duman and DeVries (118) noted that their data on the genetic difference supported observations by Hubbs (223) that differences existed among the coastal populations. Hubbs (223) delineated three subspecies, *archolepis, insignis* and *purpurescens*, and later, Peden (343) elevated *insignis* to specific status. Yoshiyama and Sassaman (528) examined seven enzymes (11 presumptive loci), two meristic characters and one morphological character in *A. purpurescens* and concluded that subspecific recognition was unwarranted for *A. purpurescens*, however, they did not cite the work of Duman and DeVries (118). Additional study of *A. purpurescens* may be needed.

GEL STAINING TEMPERATURE

Most gel staining is conducted at 37°C (329). Duplicate gels stained at different temperatures (one being the same as the acclimation temperature) may provide interesting results. Aleksiuk (6) used gel staining temperatures of 10 and 35°C and found differences in LDH between the two temperatures for red-sided garter snake, *Thamnophis sirtalis parietalis*, and eastern garter snake, *T. s. sirtalis*. Ten degree Celsius gels were stained until at least one band was as dark as the darkest band on 35°C gels. All garter snakes had been previously acclimated to 27°C, 12L:12D photoperiod and fed an *ad libitum* diet for 3 weeks. The five LDH were nearly identical in mobility and intensity in both subspecies at the 35°C staining temperature, whereas at 10°C significant differences were observable. In general, LDH 2 and 5 were strong and LDH 1, 3 and 4 were weak in *T. s. sirtalis*, whereas in *T. s. parietalis* LDH 1 and 2 were most pronounced and LDH 3, 4 and 5 were only lightly stained [see Fig. 9 in (6)]. Mester *et al.* (317) acclimated *Misgurnus fossilis* to 0 and 20°C and also stained gels at the two temperatures. Serum LDH exhibited four bands at 20°C staining temperature regardless of acclimation temperature, whereas gels stained at 0°C exhibited two LDH (warm-acclimated fish) or one LDH (cold-acclimated fish). Hoskins and Aleksiuk (216) studied the effects of acclimation temperature (4 and 20°C) on sMDH of *Thamnophis s. parietalis*. Two sMDH isozymes could be resolved in all individuals from both temperature groups; however, staining temperature had an effect on visualization on PAGE gels. Both isozymes were stained at 35°C, whereas only one appeared at 5°C. Tsukuda (461) used 18, 28 and 35°C gel staining temperatures for liver LDH from *C. auratus* acclimated to 18 and 28°C. Gel staining temperature affected the quantitative expression (activity) of LDH 1, 2 and 3 in 28°C acclimated fish only. Brush (49) investigated the effects of gel staining temperature on isozyme patterns between active and torpid bats (*Eptesicus fuscus*). No differences were observed between 4 and 37°C staining temperatures for any of the enzymes examined (LDH, EST, ALP, G6PDH, ADH, CAT, MDH and XO). Staining gels at both cold and warm temperatures may reveal additional bands of activity not present at the warm temperature due to thermal instability or inactivation and possibly may reveal what would otherwise be considered a null allele. Heat treatment of gels before staining or heat treatment of extracts before electrophoresis can also indicate thermostability differences (89,281,282,494).

MULTIPLE ELECTROPHORETIC TECHNIQUES

McLellan *et al.* (312) found one-dimensional gel electrophoresis more sensitive than two-dimensional gel electrophoresis in separating human HB of known sequence and in distinguishing among G3PDH from seven species of *Drosophila*. McLellan and Inouye (313) used 14 myoglobins of known sequence to compare various methods of protein separation, including denaturing and nondenaturing IEF and nonequilibrium gel electrophoresis. Nondenaturing IEF and nonequilibrium gel electrophoresis were shown to possess similar resolving power. They also found that nondenaturing IEF was more discriminate than denaturing IEF. However, a variety of separation techniques would provide the best overall ability to distinguish allozymes. Many more genetic differences can be revealed by varying several aspects of gels, buffers or other running conditions [see (89) for review; (11,50,91)]. The use of sequential electrophoresis has elevated levels of heterogeneity within classes of "electromorphs" in many groups of organisms (11,90). An added measure performed by Coyne *et al.* (90) was to run putative variants side by side to verify that they truly differed; this method eliminates possible error from measurements alone. However, apparently unique allelic products separated by gel sieving can also be due to PTM involving modifier loci [see (140)].

Dimmick and Page (113) used three buffer systems to resolve differences in LDH B_4s of several percid genera; the mobilities were affected differentially among the species with a given buffer. Similarly, Rose and Wilson (387) found widely different migration patterns for peafowl LDH between samples electrophoresed with buffers of pH 7.0 and 8.6. The LDH from some tissues had reversed mobilities in different buffers and the relative mobilities differed among the buffers as well. Increased heterogeneity was noted for conalbumin electrophoresed in boric acid–sodium hydroxide buffer as compared with Tris–citrate and phosphate–citrate buffers (342). The observed differences with the boric acid buffer were postulated to be possibly due to a PTM; however, no definitive evidence was presented. Murphy *et al.* (329) also noted that different buffers may affect relative mobility and isozyme activity (their Fig. 15). Brush (50) encountered greater resolving power for avian egg

white proteins using a barbital buffer rather than a borate buffer. Also, presumably homologous proteins based on electrophoretic migration could be separated immunologically. Gillespie *et al.* (152) discussed the combined use of allele and genotype data obtained from the various running conditions used in a sequential electrophoretic study to uncover cryptic variation. The method of Gillespie *et al.* (152) would be particularly useful in population comparisons. Chagnon and Guttman (70) detected cryptic variation at the *Pgm-2* locus in *Gambusia holbrooki* by examining the effects of various Cu concentrations on PGM-2 zymograms. Isoalleles were detected due to the differential effect of Cu on the two alleles. Both homozygotes and heterozygotes were detectable based on the magnitude of the effect.

SDS-PAGE is widely used to determine molecular weights of proteins or polypeptides. Huriaux and Focant (230) demonstrated a marked effect of buffer pH on the migration of carp skeletal muscle MLC. A pH effect in SDS-PAGE is very unexpected; the effect was exerted only for carp MLC_3 and not for other carp MLC, pike MLC or rabbit MLC. The difference in mobility for carp MLC_3 between two buffers altered its position relative to MLC_2 such that with one buffer its molecular weight appeared larger than MLC_2, whereas with the second buffer, MLC_3, appeared smaller than MLC_2. Huriaux and Focant (230) discussed possible explanations for the observed pH effect.

SAMPLE SIZE

As in many fields, sample size is a major concern, and biochemical systematic (electrophoretic) studies are no exception. Many recommendations have been given regarding sample sizes, and the range of reported numbers actually used has varied from one or two in some cases to over 1000 individuals (12). Grudzien *et al.* (167) examined 26 species of Goodeidae and used only one specimen of each species (using 28 presumptive loci), whereas Feder *et al.* (129) used 7580 *Gambusia holbrooki* for investigating differences within a single reservoir that contained a variety of distinct thermal habitats. Sometimes within a study sample sizes vary greatly, for example, 2–144 in MacDonald (292). The widely cited paper by Gorman and Renzi (162) indicated that sample sizes of 8–12 individuals would provide heterozygosity estimates close to those from much larger samples and that one or two individuals will usually give an estimate within 2.5% of those derived from larger samples. "Genetic distance measurements are hardly affected by sample size. A single individual may be used to represent a species for interspecific comparisons" (162). Archie *et al.* (12) re-examined the study of Gorman and Renzi (162) and found that "sampling small numbers of individuals per taxon can drastically affect the stability and accuracy of the derived pattern of relationships." In some cases a small sample size will have little or no effect, but one would only be guessing and would risk arriving at a "false" conclusion. Archie *et al.* (12) recommended that a sample of 10 or more individuals (preferably 20) be used for the most accurate phenograms derived from genetic distance estimates. The number of loci examined is also a very important aspect of an electrophoretic survey, and if constraints are imposed (financial, time), one should maintain a high number of loci (>25) and reduce the sample number of individuals (274). Investigations at the intraspecific level require a greater number of individuals to detect differences. Leary and Booke (274) and Richardson *et al.* (377) indicate that 40–100 individuals are routinely used for population studies.

INHERITANCE STUDIES

Inheritance studies are a critical but often neglected part of genetic research. Inheritance studies are needed for determining isoloci, null alleles, atypical expression and assisting in the detection of post-translational modifications (472). "Understanding the heritable basis of some electrophoretic phenotypes is sometimes impossible without inheritance data, particularly in species, such as salmonids, with a high frequency of isoloci" (472).

DEPOSITION OF VOUCHERS

The ability of future investigators to make useful comparisons with past research or to check the validity of a study may depend on the presence of some form of voucher material housed in a curated collection at an institution. Lee *et al.* (277) stated "The scientific integrity of a research publication depends on the ability of subsequent investigators to repeat the study described. Thus, the identification of the organism(s) studied is the first step in communicating the results of a biological investigation." Deposition of voucher material is common practice among taxonomists and systematists conducting morphological and meristic analyses. Publications normally list the location of materials that were examined and their respective catalog numbers. This allows a future researcher to re-examine the same specimens. The practice of taking vouchers has been and continues to be a neglected practice among biochemical systematists, population geneticists, physiologists and other investigators using organisms in their research as indicated by the available literature. Koehn and Rasmussen (263) and Scheel *et al.* (395) were early workers who preserved study material from karyological and electrophoretic studies. Buth (54), Dessauer *et al.* (109) and Cann *et al.* (68) explained the logic in retaining vouchers from molecular studies, but from examining the literature to date it appears that Buth, co-workers and a few others have followed these recommendations (52,57–59,61–63,79,112,116,120,135,161,173, 247,251,265,280,324,328,367,417,441,506,527). Karlin and Rickett (247) and Warren *et al.* (490) deposited both frozen and preserved voucher specimens. Rogers and Cashner (384), Wiley and Titus (507) and Warren *et al.* (490)

kept whole specimens and carcasses from their allozyme studies. Ferris and Whitt (137) deposited representatives of each of the species used for karyological analysis. Davis (101) retained the shells of bivalve molluscs that were used in allozyme studies; of course, there is obvious value in doing so when working with molluscs, the taxonomy and systematics of which rely heavily on the characteristics of the shells.

Buth (54) gave several reasons for the lack of voucher material: preserved (fixed) museum specimens are unsuitable for protein electrophoresis studies, after dissection for electrophoresis only a mutilated specimen may remain and investigators lacking the "systematics background" may not realize the importance of collections for documentation. Buth (54) then suggested three solutions to this problem, and I add a few more: (1) construction of depositories for frozen tissues; (2) careful dissection of specimens to minimize physical damage; (3) collection of a number of specimens that exceeds the number required for protein electrophoresis; part of the sample will be used for the allozyme/isozyme study and part will be fixed and deposited in a museum collection; (4) preservation of fish remains in ethanol; although only a carcass may remain after tissues have been dissected, useful data remains. If some specimens or tissues are preserved in ethanol, DNA may be extracted in the future which may provide complementary or contradictory results to the previous study (109). Also, if an individual specimen exhibited strange variation (phenotypes on zymograms) one could examine the fixed remains for parasites or the possibility the specimen was a hybrid that was overlooked after collection. (5) Skeletal elements can be diagnostic; specimens (even badly dissected) preserved in formalin retain valuable meristic data as well as the skeleton that can be visualized by clearing and staining. Also, skeletal preparations could be made of some fresh specimens using dermestid beetles or enzyme digestion methods. Lee *et al.* (277) suggested among other things that scientific societies and/or publishers of scientific journals refuse publication of a manuscript unless voucher material has been properly documented according to established criteria. Deposition of voucher material from molecular studies demands as much attention as from morphological studies; this practice can only strengthen the field of molecular systematics and also, help maintain stability in the ever-changing taxonomic assignment of species.

ECOGENETICS AND ECOPHYSIOLOGY

Many species exhibit differences in thermal preferences or CTMax based on prior exposure temperature, season in which captured, disease or pollution [(66,87,198,204,206, 268,289) and references therein, (469)]. *Luxilus cornutus* captured in fall had a lower temperature preference than those captured in spring (469). Three species, *Notropis stramineus, Luxilus cornutus* and *Etheostoma nigrum*, exhibited seasonal differences in CTMax, even though all groups had been acclimated to the same temperature and photoperiod for 4 weeks before testing (268)!

Considering the number of individuals used for the CTMax determinations, it is unlikely that genetic differences were responsible for the observed seasonal variation. The results of Kowalski *et al.* (268) suggest that acclimation periods should be at least 4 weeks or greater or that all test organisms should be captured within a narrow time span to eliminate seasonal effects [e.g., (478)]. Bulger and Tremaine (51) determined CTMax for *Fundulus heteroclitus* captured at different times of year and acclimated in the laboratory to similar natural temperature and photoperiod at the time of capture. Midday CTMax varied seasonally in *F. heteroclitus*. Fishes captured in November and acclimated to summer (July) temperature and photoperiod were tested for CTMax at 6 and 12 weeks postcapture and at both times the CTMax was lower than the normal summer midday CTMax. Therefore, some seasonal effects apparently remained even after 12 weeks of acclimation! Seasonal differences in splake and brook trout IDHP seemed to remain even after temperature and photoperiod acclimation times of greater than 1 month (321). Bacterial infection in *C. auratus* caused fishes to increase their preferred temperature, a phenomenon known as a "behavioral fever" (87). Experiments are needed that will examine the time required to "override" the effects of prior exposure conditions. Richards *et al.* (376) provided recommendations regarding thermal preference and thermal tolerance experiments. Temperature, seasonal and diurnal effects (photoperiod); social effects; age/size of test fishes and feeding schedule were some of the variables mentioned by Richards *et al.* (376) that would also apply to electrophoretic research. Oxygen content should also be controlled because this has been shown to affect the final preferred temperature of *O. mykiss* (401). Considering that most species experience gradual changes among the seasons, it seems likely that some underlying biochemical changes may also occur rather slowly and that their effects disappear slowly as well even though an organism has apparently adapted to the new exposure conditions. Terpin *et al.* (450) mentioned that light intensity may be important in evoking a response; light intensity should be measured and reported if possible [e.g., (379)]. *Fundulus heteroclitus* exhibited seasonal variation in brain ChE activity as measured over a 5-month period even though the fishes were held at 13 ± 2°C for the entire time; photoperiod control, if any, was not discussed (21). Effects of photoperiod are likely manifested through the photosensitive pineal gland. Diel changes in behavioral thermoregulation by *Catostomus commersoni* have been shown to be regulated by the pineal gland (250). Reproduction in most vertebrates is also regulated to some degree by photoperiod (372).

Thermal tolerance can also vary intraspecifically as well due to differences in local environments (131,306). Photoperiod also appears to have an effect on the upper incipient

lethal temperature (450). Photoperiod exerts effects on fish physiology independent of temperature. The optimum temperature for growth of *Morone saxatilis* ♀ × *M. chrysops* ♂ was lower (25.7°C) when held at an autumnal photoperiod (decreasing day length) as compared with 27.9°C under a spring photoperiod (increasing day length) for certain ration sizes (516). Growth and salinity tolerance of juvenile *Salmo salar* were affected by photoperiod as well (390).

The regulation of temperature and even photoperiod in acclimation studies may not be enough. Kleckner and Sidell (255) found some enzymes to vary widely between naturally acclimatized winter and summer captured *Esox niger* and laboratory acclimated groups of 5 and 25°C (all 12L:12D photoperiod and fed live fishes). The changes in enzyme activities were expected to be very similar for the 5°C fishes and winter fishes and for the 25°C fishes and summer fishes. For some enzymes (LDH), the laboratory-acclimated groups were similar to each other, whereas differences existed between the acclimatized groups. CYTOX activity from brain was not significantly different between naturally acclimatized fishes but was higher in 5°C fishes than in 25°C fishes. LDH activity was lower in 5°C fishes than 25°C, but the opposite was true for the naturally acclimatized fishes. Some enzymes in some tissues did respond as expected, however. Kleckner and Sidell (255) suggested that differences in photoperiod, diet and swimming activity may have been responsible for the observed variation in activities between laboratory-acclimated and naturally acclimatized fishes. The activity of CYTOX has been shown to be related to diet (feeding vs starvation) (217).

A smaller ration size caused *S. namaycush* to select a lower final preferred temperature (291). Preferred temperatures of *Salvelinus fontinalis*, *Salmo salar* and *Oncorhynchus mykiss* differed by several degrees Celsius between fed and starved fishes (234). Smaller ration size lowered the optimum growth temperature for *Morone saxatilis* × *M. chrysops* (516). Houlihan *et al* (217) indicated that most temperature acclimation studies on growth or physiology of fishes provided food *ad libitum*. Some studies entailed feeding on a daily basis for warm groups and every other day for cold groups, probably because the cold group simply did not consume as much food (95,145,406,411,414). Houlihan *et al.* (217) recommended the use of similar ration sizes between/among experimental groups. This seems to be a good idea; however, consumption may naturally differ among groups, especially between extreme temperatures [e.g., (216)]. Jobling (236) stated: "Water temperature will have a major influence on the amount of food consumed by a fish." Levels of dissolved oxygen may also affect food consumption (236). Prosser (359) was aware of potential problems in establishing feeding regimes as well as control of other variables:

"A serious problem, especially with poikilotherms, is the identification of appropriate environmental variables. Three factors, temperature, nutrition, and photoperiod, interact in an inextricable way. Many fish and amphibia eat little in the cold, and it has been common practice to observe acclimation in starved animals. Unfortunately a fish starved at 25°C is not comparable in its food reserves to one starved at 5°C. Also if each is fed *ad libitum*, the absorption of food may be so slow in the cold that the nutritional state is different from that of one fed at 25°C."

Blier and Guderley (34) varied diet between 5 and 18°C groups of *Coregonus clupeaformis* to maintain similar body mass between the two groups and avoid variation due to body mass differences. Van den Thillart *et al.* (451) used ration sizes of 1.55, 0.58 and 0.30 g per 100 g body weight at 20, 10 and 5°C groups, respectively. *Salvelinus fontinalis* acclimated to warmer temperatures and fed to satiation have larger livers than conspecifics held at colder temperatures (521). Dewaide (110) found larger livers in cold-acclimated (5°C) vs warm-acclimated (18°C) *O. mykiss* and *Leuciscus rutilis*. Kolok (264) acclimated *M. dolomieu* and *L. cyanellus* to 5, 15 and 25°C for 10 weeks and fed the 15 and 25°C groups thrice per week and the 5°C group once per week. *M. dolomieu* held at 5°C did not feed, which is consistent with natural observations for this winter-quiescent species (264,383). The green sunfish remains active and feeds at 5°C; therefore, the natural differences between or among species must be dealt with in some way. In such a case, the lack of feeding in one group may mask or exaggerate observed trends. Of course, such a difference would be present if examining naturally acclimated organisms. Crawshaw (94) reported very low food intake by *M. salmoides* acclimated to 4, 6, or 8°C, and food intake increased with rising temperature (tested up to 18°C). *Cyprinus carpio* held at 2 and 5°C progressively decreased their food intake after one week at these temperatures and completely ceased feeding after 3 and 6 weeks, respectively (95).

Although the differences in feeding activity may affect measured enzyme activity, if a study is intended to examine true seasonal differences, an *ad libitum* feeding regime may be adequate. However, to isolate temperature effects, similar ration sizes would be more appropriate. One must also realize that the food resources available to fishes may differ in quantity and type among streams, lakes and ponds and may differ temporally as well. So what is a natural feeding regime? Because the natural diet can be so variable, holding experimental animals in the laboratory and supplying a standard diet may reduce variability. In summary, the feeding regime used depends on the objectives of the research and if appropriate, should consider the species' natural foods and consumption rates, both daily and seasonally.

Critical oxygen tension may also change seasonally with some species having a lower critical oxygen tension at warmer (20°C) temperatures (205,468) and others exhibiting the converse (205). As oxygen tension is reduced, *O. mykiss* selects lower temperatures (401). Differences in oxygen consumption have been demonstrated between spring- and summer-captured *Pseudacris triseriata* even after a 5- to 7-day acclimation period [Dunlap, 1980, in (83)].

Simply because a species consistently selects a"preferred temperature" in the laboratory does not mean that species will be found at that temperature often in the wild. Thermal habitat is a commodity just as are structural habitat, water velocity and food (294). Organisms must compromise preferences for some resources such as food (or temperature) when competition exists either intra- or interspecifically (294). However, studies have shown that the final temperature preferendum of a fish in the laboratory often coincides with maximization of at least some physiological parameters [e.g., (47), references in (86)].

Some species of fish (*Ameiurus natalis* and *Morone saxatilis*) demonstrate an ontogenetic shift in thermal niche (86,374). It would also be interesting to study the biochemical changes that may be associated with this phenomenon. *Morone saxatilis* appears to be a species with very strict oxygen and thermal requirements based on the work of Coutant (85,86). In freshwater reservoirs, *M. saxatilis* adults often die during summer when they are "squeezed" into a narrow zone where their preferred temperature and sufficient dissolved oxygen can be found. The fishes do not venture from their thermal refugia even for food and as a consequence are often found overcrowded, emaciated and diseased. It is puzzling that one or two requirements must be adhered to so strictly, whereas another critical need (feeding) seems to be "ignored." Possibly in its native habitat (coastal ocean), the need to compromise was not necessary, and it has evolved into a very stenothermal species. Certainly, a biochemical study of *M. saxatilis* enzyme functional constraints would complement the previous ecological studies of Coutant and colleagues. Genetic constraints as additional factors in the ecology of organisms have not been frequently investigated [e.g., (201,315,347,357)].

The observation that *Ameiurus nebulosus* acclimated to 10 and 20°C did not differ with respect to electrophoretic mobilities for 17 enzymes (+EST and PROT) implies that these enzymes may be eurythermal and can function over a wide temperature range (430). The results of Somero (430) may explain the rapidity at which cold-acclimated *A. nebulosus* select much warmer temperatures (93). *A. nebulosus* acclimated to 7, 15, 24 and 32°C for 10–20 days initially selected temperatures of 16, 21, 26 and 31°C, respectively, and most had selected temperatures between 29 and 31°C (the final thermal preferendum) within 24 hr (93)!

The most important aspect of an enzyme is its functionality, and specific amino acids (e.g., those at the active site) are more important in maintaining enzymatic function than others. Therefore, conservation of function (which is most important) (214) can be achieved through retainment of the functionally important residues. For example, nine amino acids in the HB molecule have remained unchanged in 60 species ranging from lampreys to humans; these residues are critically important for the function of the HB molecule (442). "The outcome of adaptation . . . is not homeostasis (the same state) but is better termed enantiostasis (conserved function), a concept first formalized by Magnum and Towle (1977)" (214). Hubbs (224) stated "Environment-related problems can further plague taxonomy because survival in similar environments tends to select for similar morphologic attributes. . . . Practically, one must be careful not to use apparently divergent characters that happen to have selective value in similar habitats. Use of 'non-adaptive' characters for taxonomy should help resolve problems of this type, but can one be certain that any character is not adaptive? . . . Even this 'ultimate' systematic tool [electrophoresis] may have potential weaknesses."

An example by Hubbs (224) that seems to be supported based on work by Graves and Somero (163) is as follows: "Assume that we have two ancestral species of omnivorous fishes occupying estuaries. Both evolve into a freshwater herbivore and a saltwater carnivore. The DNA sequences in each species pair would diverge so that each saltwater type would have a sequence of codings favorable for survival in high salinity. Similarly each would have their DNA controlling their digestive enzymes designed to break down animal material. The freshwater representatives of each pair would have their nucleotide sequences designed to produce enzymes different from their sibling species, but the same as those of their more distantly related ecological counterpart. The above simplified model is undoubtedly extreme, but may indicate how dependence on a single analysis could be hazardous."

The above hypothetical example brings up an interesting situation, but could such an occurrence have any possibility of happening? Research on barracudas (*Sphyraena* spp.) by Graves and Somero (163) suggest that such events may be possible. They found greater divergence in LDH A_4 between more recently separated and more proximally located species that occupied different thermal habitats than was found between more distantly (spatially) and temporally separated species that experienced similar thermal regimes. The two species separated longer in time and by greater distance, *S. argentea* and *S. idiastes*, had LDH A_4 of identical electrophoretic mobility and kinetic properties [see Fig. 11–13 in (214)]. All the barracudas are otherwise very similar in morphology and ecology. Conversely, sMDH from the same four species of barracuda possessed similar kinetic properties (e.g., K_m vs temperature) and had identical electrophoretic mobilities, but the tropical species, *Sphyraena ensis*, did not express the thermolabile sMDH that occurs in the other three *Sphyraena* spp. and in most teleosts (281). In species pairs of the genera *Thalassoma* and *Ophioblennius* from Atlantic and Pacific waters, LDH A_4 of species from the Atlantic were more similar kinetically to each other than to the orthologous enzyme from Pacific species (164). Atlantic waters along the Panama coast have temperatures an average 2–3°C higher than adjacent Pacific waters and are less variable over time as well. Only in the case of *Thalassoma* spp.

were the LDH both kinetically and electrophoretically distinguishable; in *Ophioblennius* spp. the LDH were distinguished kinetically, but not electrophoretically (164).

Baltz *et al.* (20) investigated the importance of eight microhabitat variables to four fish species in a northern California stream and found in part that "In a situation where stream fishes were able to select temperature as well as other environmental features, temperature proved to be a better predictor of where each species was found than were two of the other three variables (i.e., mean water column velocity and substrate) most commonly used in instream flow models." Hochachka and Somero (214) stated that "Body temperature, not phylogenetic status, is the dominant component in establishing the relationships of enzymes to temperature." McDonald (309) remarked that "Since then, more evidence has accumulated suggesting that protein-coding loci may, in many instances, be relatively indifferent to speciation and other macroevolutionary events. . . ." Slatkin (419) indicated that "Natural selection can be much more effective than genetic drift in either preventing or establishing local differences. Selection in favor of the same alleles or the same traits would produce geographic uniformity regardless of any green flow. . . . The absence of gene flow. . . . does not necessarily trigger rapid evolution; salamanders in particular have evolved very little in their long history. . . ."

Therefore, it appears that evolutionary trends cannot be based on random proteins and that comprehensive study of various aspects of a protein must be conducted across many groups of organisms. Additionally, one should look at variation in environmental conditions between or among groups of organisms and consider there potential influence on genetic similarity (possibly due to convergence) or differences. Ecological factors and their effects on protein evolution need to be considered in phylogenetic analyses based on molecular data sets since convergence due to environmental constraints has been demonstrated [also see (434)].

CONCLUSION

Shaklee *et al.* (406) stated that thermoacclimatory responses of fishes may not involve major isozymic changes. However, the knowledge of temperature–isozyme relationships in fishes to date indicates that although most species thus far examined exhibit some quantitative changes in their isozyme complement, some species have been shown to possess "inducible" isozymes depending on ambient environmental conditions. The multiple variant enzyme system has been found in a variety of plant and animal species, therefore making the assumption that no qualitative changes are a possibility may produce erroneous results in taxonomy, systematics or population genetics. If individuals among samples possess a multiple variant system, environmentally induced variation might be construed as fixed, genetic variation based on the interpretation of electrophoretic zymograms. If the situation with *O. mykiss* brain AChE was present, comparisons from intermediate temperatures (which would possess two bands) and from either extreme warm or cold temperatures (which would possess only one band) would indicate genetic variation when in fact no genetic variation was present. Another possible situation involves the presence of only one or two individuals within a population that are expressing an environmentally induced isozyme change. These individuals may be classified as possessing a mutant allele and not considered further. Also, temperature-mediated lipid-protein interactions or lipid changes may cause alterations in an enzyme's activity and possibly its mobility (82,181,182,186,190,386).

Only a handful of species (and within them, only a small number of enzymes) have been studied for acclimation effects on isozymes and allozymes. Extrapolating these results to all species and assuming no effects will be present is unrealistic considering the total number of species in existence and the demonstrated prevalence of "inducible isozymes" (Table 1). Therefore, making the general assumption that thermally inducible isozymes do not occur, except in *O. mykiss* and a few other species, is not advised.

A solution would be to acclimate specimens to the same environmental conditions before collecting the tissues to be used (which would require live transport, holding facilities and increased time and monetary expenditures) or temperature and other data could be collected in the field with samples being collected only within a narrow range of temperature. One must then be able to show that the field conditions have been stable for 3–4 weeks, which is unlikely, before the collection of specimens and recording of environmental parameters because conditions at the time of capture may not be indicative of recent conditions and the organisms may not be conditioned to these new ambient conditions. Even if none of the previous steps are taken, temperature measurements would be valuable (377) to future investigators conducting systematic or acclimation studies.

Only further detailed studies on individual species will elucidate which exhibit complete, environmentally inducible isozymes; Johnston (240) indicated the need for more thorough research on qualitative and quantitative variation in enzymes and proteins. Procedures should be implemented that control for temperature, pH, photoperiod, feeding schedule, dissolved minerals and other parameters of water that may contribute to isozymic variation. Other sources of potential variation discussed earlier should also receive more attention in the future. These procedures will require higher expenditures of both time and money for investigators, but the return will be more reliable data for inferring relationships among organisms and for physiological studies in general. I hope this review will stimulate further work concerning plasticity of gene/enzyme systems, the enzy-

matic changes associated with environmental stimuli and altered physiological states and, specifically, how these changes affect interpretation of protein electrophoretic data used in taxonomic, systematic and population genetic research. "The question still remains whether altered environmental temperatures induce dramatic alterations in isozyme patterns in some fish" (204).

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