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REVIEW

Nongenetic Variation, Genetic–Environmental Interactions and Altered Gene Expression. II. Disease, Parasite and Pollution 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, there are many confounding factors that may alter the results of an electrophoretic study and may possibly allow erroneous conclusions to be drawn in taxonomic, systematic or population studies. Measured enzyme activities can also be affected significantly. Parasites, disease and pollution can affect levels of enzyme activity, and electrophoretic results can be affected both quantitatively and qualitatively. Blood serum is particularly vulnerable to variation due to disease, pollution or parasites because damaged tissues may release tissue-specific enzymes into the bloodstream. Capture, handling, chemical treatments, bacteria, natural toxins and consumed food may also contribute to variation. Potential pollution impacts at specimen collection sites should be investigated, and study organisms should be inspected and/or treated for detection and elimination of parasites and disease. COMP BIOCHEM PHYSIOL 117B;1:61–74, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Isozymes, allozymes, acclimation, heterogeneity, inducible isozymes, artificial selection, allele frequency, metals

INTRODUCTION

Protein electrophoretic techniques have been, and continue to be, widely used and accepted tools in systematic and population studies (6,26,44,175,176,192). However, there are many factors that can affect the results of an electrophoretic study or measurements of enzyme activity (133,134). Serious attention to known problems with electrophoresis has been lacking in the past, but some investigators have expressed concern (3,81,112). The possible ramifications of such variation with regard to systematics has rarely been considered (17,22,111,140). Some investigators have addressed briefly how these changes may affect interpretations and conclusions (51,85,99,100,126).

The number of isozymes and allozymes (= multiple staining bands on a gel) expressed has been shown to be affected by temperature, diet, pH, photoperiod, sex, female reproductive state, posttranslational modifications, sample processing procedures, experimental methodology, storage time, pollution, disease, parasites and other stressors [see (133,134)]. The purpose of this review is to examine the effects of pollution, parasites, disease, bacteria, consumed food and capture/handling stress on electrophoretic phenotypes and discuss methods that will help avoid such problems. Nomenclature of organisms is given in ref. 133. Abbreviations for enzymes generally follow Shaklee *et al.* (151) and Murphy *et al.* (112), and enzyme names and enzyme commission (EC) numbers are those recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (76b). Following is a list of enzyme and protein abbreviations used: aspartate aminotransferase (or aspartate transaminase, AAT) and mitochondrial aspartate aminotransferase (mAAT), EC 2.6.1.1; alanine aminotransferase (or transaminase, 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; α-amylase (AMY), EC 3.2.1.1; porphobilinogen synthase (δ-amino levulinic acid dehydratase) (ALA-D), EC 4.2.1.24; catalase (CAT), EC 1.11.1.6; creatine kinase (CK) and mitochondrial creatine kinase (mCK), EC 2.7.3.2; cytosol aminopeptidase (CAP), EC 3.4.11.1; enolase (ENO), EC 4.2.1.11; epoxide hydrolase (EH), cytosolic EH (sEH), EC 3.3.2.3; esterases (EST), EC 3.1.1._; fructose-bisphosphate aldolase (FBALD), EC 4.1.2.13; glucose 1-dehydrogenase (GDH), EC 1.1.1.47; glucose-6-phosphate 1-dehydrogenase (G6PDH), EC 1.1.1.49; glucose-6-phosphatase (G6Pase), EC 3.1.3.9; glucokinase (GK), EC 2.7.1.2; glutathione reductase (GR), EC 1.6.4.2; glucose-6-phosphate isomerase (GPI), EC 5.3.1.9; glutathione peroxidase (GPX), EC 1.11.1.9; glutamate dehydrogenase (GTDH),

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EC 1.4.1.2; glutamate dehydrogenase, $NADP^+$ (GTDHP), EC 1.4.1.4; 3-hydroxybutyrate dehydrogenase (HBDH), EC 1.1.1.30; heat shock proteins (HSP); hemoglobin (HB); isocitrate dehydrogenase, $NADP^+$ (IDHP), EC 1.1.1.42; L-lactate dehydrogenase (LDH), EC 1.1.1.27; malate dehydrogenase (decarboxylating, NAD^+) (or malic enzyme, ME), EC 1.1.1.39; mannose-6-phosphate isomerase (MPI), EC 5.3.1.8; metallothioneins (MT); unspecific monooxygenase (mixed-function oxidases, MFO), EC 1.14.14.1; ornithine—oxo-acid transaminase (ornithine aminotransferase, OAT), EC 2.6.1.13; ornithine carbamoyltransferase (OCT), EC 2.1.3.3; peroxidase (PER), EC 1.11.1.7; phosphogluconate dehydrogenase (decarboxylating, PGDH), EC 1.1.1.44; phosphoglucomutase (PGM), EC 5.4.2.2; pyruvate kinase (PK), EC 2.7.1.40; superoxide dismutase (SOD), EC 1.15.1.1; succinate dehydrogenase (SUDH), EC 1.3.99.1; tyrosine transaminase (formerly called tyrosine aminotransferase by some, TAT), EC 2.6.1.5; xanthine oxidase (XO), EC 1.1.3.22. Other abbreviations: isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE), carbon tetrachloride (CCl_4), copper sulfate ($CuSO_4$), methyl mercury (MeHg), mercury nitrate ($MgNO_3$).

DISEASE

The use of biochemical "markers" or biomarkers to monitor and detect stress or disease may be valuable for monitoring pollutants in the environment (see Pollution). The possible role disease might play in relation to enzymes, besides alterations that are genetically based, is that damaged tissues can "leak" enzymes into the surrounding body fluids (83,92,108,158,179). Healthy cells would most likely not import any of the "leaky" isozymes because their cell membrane would be functioning normally. Enzymes can be transported across membranes, however. For example, most mitochondrial proteins are encoded by nuclear genes, manufactured in the cytoplasm and transported into mitochondria (202). In clinical medicine, blood serum and other fluids are often monitored for detection of disease that results in cell/tissue damage (32,92,158,167). Increased blood levels of AAT and LDH can indicate liver damage in humans and increased levels of AAT, CK, LDH and HBDH occur after myocardial infarction (necrosis of heart tissue) (83,108,129,167,179,182,195,197). Cytosolic AAT may leak from liver with minor damage, whereas mAAT does not leak into the bloodstream until more extensive damage has occurred (182). Similarly, the appearance of mCK in blood indicates more severe tissue damage (167). Clinical applications of LDH isozymes were covered by Vesell (179), Skillen (158) and Sun (167). Danpure (32) reviewed the use of LDH as an indicator of cell injury in tissues of heart, liver, kidney and lung; injury is monitored through levels of LDH present in various bodily fluids. Vesell (179) listed 22 enzymes and Kaplan *et al.* (83) listed 14 enzymes commonly used in clinical medicine, and most enzymes are routinely included in electrophoretic studies. Two volumes of *Clinics in Laboratory Medicine* contain a wealth of information concerning the use of isozyme patterns for detecting diseases (129,196). Enzymes that have been shown to be diagnostic of disease states in humans based on either quantitative or qualitative changes are LDH, AAT, AMY, CK, ALAT, ENO, ALP and CAP (42,129,145,167,196,197). Increased amounts of AAT, ALAT, CK, LDH and MDH were detected in blood serum of dogs after experimentally induced myocardial infarction, whereas EST gel patterns remained unchanged (49). In humans, serum CK levels were highly elevated due to muscle biopsy, intense weight-training and injury to leg muscle (hamstring) incurred while running (70).

Cancer has also been associated with quantitative and qualitative isozyme changes in LDH (149 and references therein), PK, FBALD (147) and qualitative changes in AAT (37). Often, the observed isozyme changes resemble a regression to fetal isozyme patterns (147). Serum LDH and CK patterns may differ quantitatively in humans with muscular dystrophy (167,194). Aberrant gene expression may be caused by muscular dystrophy (184), and muscular dystrophy is apparently not uncommon in fishes, amphibians and reptiles (165). Quantitative differences in percent composition of LDH isozymes have also been demonstrated between normal and dystrophic muscle in humans (194). An additional LDH isozyme, LDH-6, occasionally appears cathodal to LDH-5 in human patients; many of these patients die soon after this LDH is "expressed" (197). Immunological analysis proved the LDH-6 contained M subunits, and the enzyme was very stable. LDH-6 may be a posttranslational modification of LDH-5 or an ADH (197).

Marquez (104) examined several enzymes in serum of non-spawning, pre-spawning and spawning pink salmon (*Oncorhynchus gorbuscha*). Significant differences in activities of LDH, AAT, CK and HBDH were found between the non-spawning and spawning groups (5–12 individuals assayed). Higher activities found in the spawners were likened to higher activities of these same enzymes associated with some degenerative diseases in humans (104). Racicot *et al.* (136) studied the effects of *Aeromonas liquefaciens* infection on plasma enzyme levels in rainbow trout (*Oncorhynchus mykiss*). *A. liquefaciens* infection of the caudal peduncle region resulted in increased plasma activities of LDH, AAT, ALAT and CK. Generally, the more severe the infection, the greater the plasma enzyme activity. The largest changes were for LDH and CK (136). Brook trout (*Salvelinus fontinalis*) infected with *Aeromonas salmonicida* had significantly elevated FBALD, CK and OCT activities at 72 hr post-injection (154). Atlantic salmon (*Salmo salar*) infected with *A. salmonicida* had significantly lower total serum protein levels than non-infected salmon, and mean protein levels were lower in more severely afflicted individuals. Quantitative changes in serum proteins also were present between healthy and diseased salmon (109). In some

cases, the appearance of new "isozymes" after bacterial infection may be due to proteolytic modification (or other posttranslational modifications) of host enzymes because, at least for *A. salmonicida*, proteases and phospholipases are secreted by the bacterium (76a,135). Serum from *Saprolegnia*-infected brown trout (*Salmo trutta*) had elevated activities of LDH (three times), AAT and ALAT (approx. eight times), ALP (four times) and ACP (two times) over healthy trout serum enzyme activities (39). Another study of *Saprolegnia*-infection in *S. trutta* found significantly elevated levels of serum ALAT, AAT and CK with the CK levels being extremely high (23). Bucher and Hofer (23) provided a summary of other similar studies. HB levels were often significantly lower in winter flounder (*Pleuronectes americanus*) with bacterial infection compared with healthy individuals (101). Blood plasma of *O. mykiss* had elevated levels of LDH-$B2_4$ when fishes were infected with infectious hematopoietic necrosis virus (4). Reichenbach-Klinke (139) indicated that qualitative variations occurred between blood serum protein patterns of healthy and diseased *O. mykiss*. Sindermann and Mairs (157) noted quantitative and qualitative differences in some serum proteins between healthy and infected (with fungus, *Ichthyosporidium hoferi*) Atlantic herring (*Clupea harengus*), and *Salmo salar* with "ulcerative dermal necrosis" had serum protein profiles that differed quantitatively and qualitatively from healthy salmon (110). Cai (28a) electrophoretically and densitometrically compared seven enzymes in six tissues of grass carp (*Ctenopharyngodon idella*) with and without hemorrhagic disease and found 10 cases of lowered activities in diseased compared with healthy fishes for various enzymes/tissues. Booke (18) also cited several early disease-related studies in his review of variations in fish serum proteins. Larvae of Japanese beetles (*Popillia japonica*) infected with *Bacillus popilliae* had both qualitatively and quantitatively different hemolymph protein patterns than non-infected beetle larvae (11). The larvae were injected with *B. popilliae*, then hemolymph was collected after 7 days and samples were centrifuged to remove the *B. popilliae*. Bennett *et al.* (11) suggested that the additional staining band in diseased beetles might be a product of proteolytic modification. Diseased beetle larvae also had a much smaller quantity of the major lipoprotein. Raymond *et al.* (138) found that β-lipoprotein exhibited altered mobilities when sampled at different times from the same human subjects.

Latner and Skillen (92) cited a number of cases in plants where disease affected the isozymes of ACP, ALP, SUDH, MDH, CAT and PER. Rudolph and Stahmann (143) found lowered staining activities of some enzymes in bean plants (*Phaseolus vulgaris*) infected with *Pseudomonas phaseolicola*. Additional PER isozymes appeared in tobacco plants after infection with *Pseudomonas tobaci* (162) and tobacco mosaic virus (90). *Fusarium oxysporum*-infected pea plants exhibited quantitative increases in CAT, ALP, GTDH and decreases in some PER, EST, ACP, GK and FBALD. In addition, novel isozymes, which were not present in healthy plants or the fungus, appeared in infected plants. Novel isozymes of PER, FBALD, GK, G6PDH, GTDHP, ME and MDH were present only in the infected plants. Possible explanations for the novel enzymes were transcriptionally or translationally controlled plant isozymes or plant–fungus subunit interactions resulting in hybrid enzymes with unique mobilities (162). Barley leaves infected with *Erysiphe graminis* possessed additional EST, ACP, MDH, SUDH and PER isozymes, and, again, none of the novel isozymes were present in either healthy leaves or fungal mycelia and conidia from infected leaves (162). Induction of low molecular weight proteins, called pathogenesis-related proteins, has also been noted for plants infected with bacteria, viruses or fungi (146).

Although enzyme alterations are markers of certain disease states in humans, the same markers may not apply for other organisms. However, it seems that enough evidence is currently available to suggest extreme caution. Changes in serum enzymes have been shown to be particularly sensitive. Organisms used in any enzyme study should be observed for any signs of disease before collection of tissues. van den Thillart and Smit (169) treated diseased goldfish (*Carassius auratus*) before using the fishes in a study of acclimation effects on enzyme activities. Perhaps all experimental animals should be treated before study regardless of any outward signs of disease, thus eliminating possible variation in results. A suitable post-treatment time would also be required to ensure that the treatment itself does not contribute to experimental variation. Thoesen (170) should be consulted regarding disease detection in fishes. Stickney and Kohler (164) covered proper laboratory maintenance of fishes.

POLLUTION

Manwell and Baker (102) suggested that biochemical polymorphisms could be influenced by pollutants, because some enzymes "interact directly with pesticides and other pollutants" (e.g., EST). Recent research has implicated various pollutants (e.g., metals, insecticides, heat) as factors influencing the genome of fishes and other organisms (12,46,47,54,75,117,118,200). The impacts of pollution on the genome can be manifested in both short-term (increased or decreased enzyme content or activity) and long-term time frames (selection effects on genome or changes in allele/genotype frequency), depending on the intensity and duration of the affecting agent. Guttman (57) discussed the potential usefulness of monitoring population genetic structure to detect pollution impacts and suggested that future work should examine both short-term and long-term effects of pollutants. Phipps *et al.* (132) provided a general review of the effects of several hundred pollutants on various aspects of accumulation, physiology and so on in aquatic organisms.

Metallothioneins, HSP, MFO and other detoxification enzymes may be useful to monitor as indicators that organisms have had prior exposure to metals or other pollutants (1,24,69,80,86,137,171). Specific inhibition of ALA-D by Pb *in vivo* may be a useful biomarker of environmental lead exposure (72). Factors such as temperature, size, age, reproductive state and nutritional status can affect MFO enzyme activities and therefore result in seasonal differences in enzyme activity (1,79,80,84,133,137). There is an immense body of literature concerning HSP or stress proteins, and the reader is referred to the following for more information (94,95,114,120,121,130,148). Nover (120) listed 113 known inducers of HSP synthesis. Waxman and Azaroff (185) reviewed induction of cytochrome(s) P450 and levels of control responsible for differences in their quantitative expression. A review of plant MT was recently published by Robinson *et al.* (141); they noted that MT expression (induction) mainly is regulated transcriptionally.

Cadmium poses serious consequences to organisms due to inhibition of protein, DNA and RNA synthesis and competition with other divalent cations (e.g., Zn, Cu, Ca) that are essential for function in some metalloenzymes (52,178). Alkaline phosphatase from trout intestine is dependent on Mg but is inhibited by Hg and Zn (191), and inorganic Hg can inhibit EST in caddisflies (46). Gill *et al.* (53) studied the *in vivo* and *in vitro* effects of Cd on AChE, ACP, ALP, LDH, AAT and ALAT in rosy barb (*Barbus conchonius*) liver, gill, kidney, heart, brain, testes, ovaries and skeletal muscle. Effects varied greatly among the *in vivo*, *in vitro* and control groups as well as among different enzymes and tissues. In several instances, *in vivo* activity was significantly different from both *in vitro* and control activities, which differed very little in comparison, suggesting that some of the enzyme responses *in vivo* may be the result of an indirect interaction rather than a direct inhibitory or stimulatory effect of the metal. Also, some observed differences may relate to levels of a metal for a given tissue, because some tissues accumulate more of a particular metal than other tissues (73). Hodson *et al.* (72) also suggested that differences between *in vivo* and *in vitro* effects of metals on ALA-D may be due to spatial factors. Significant differences between *in vivo* and control groups were found for AChE in gill (−), muscle (+) and brain (+), ACP in gut (+) and ovary (+), ALP in kidney (+), gut (−) and ovary (+), ALAT in muscle (−) and kidney (−) and LDH in heart (+) (53). Significant differences in SOD activity were found for lake trout (*Salvelinus namaycush*) and white sucker (*Catostomus commersoni*) (in some cases) between Cd-polluted and non-polluted lakes in close proximity; the same was true for CAT in *S. namaycush*, *C. commersoni* and pearl dace (*Margariscus margarita*) and GPX in *C. commersoni* (128). Cadmium initiates transcription of MT and HSP genes (59,94). Increased tolerance to metals may be, in part, due to synthesis of MT, which scavenges the metals, thus preventing disruption of some biochemical processes (73,86,98). Hogstrand and Haux (73) reviewed the role of MT in conferring protection against metals, primarily in fishes. Many enzymes are known to exhibit either decreased or increased activity due to metal or contaminant exposure (see 65,66). Hepatic ACP, ALP, XO, and CAT activities in mummichogs (*Fundulus heteroclitus*) exposed to various metals were significantly lower compared with controls (77). Such changes in activity may also be apparent after gel electrophoresis (i.e., differences in staining intensity). Regardless of whether examining physiology or genetic makeup, prior exposure of some study organisms to metals or other contaminants may introduce high levels of variability in assay or electrophoretic results. Both quantitative and qualitative differences were noted for HB and plasma proteins among four groups of moggel (*Labeo umbratus*) exposed to various toxicants compared with controls (177). Qualitatively, the control *L. umbratus* ($n = 10$) each possessed four HB, whereas all experimental groups showed only three HB using PAGE. To minimize variability, all individuals were captured from one locality at the same time and acclimated to the same conditions before use (177). *In vivo* phenylhydrazine treatment in sharptooth catfish (*Clarias gariepinus*) resulted in large quantitative decreases for mMDH in white muscle and retina, GPI in kidney and GPI, ADH and LDH in liver and total losses of activity for sMDH in white muscle and retina, ADH and LDH in kidney and LDH and GPI in retina (8). Intraperitoneal injection of benzo[a]pyrene into bluegill (*Lepomis macrochirus*) resulted in higher MFO activities, and two additional polypeptides were present on SDS-PAGE gels (79). Using SDS-PAGE, Dutta *et al.* (40) demonstrated both qualitative and quantitative changes in serum proteins of *Lepomis macrochirus* exposed to non-lethal levels of methyl mercury (MeHg). Total serum protein levels differed as did the number of polypeptides, which varied from 28 in controls to 22, 51 and 27 in MeHg-treated fishes at 24, 48 and 72 hr post-treatment, respectively (Fig. 1). The appearance of more protein bands was thought to be due to breakdown of red blood cells, HB or other intracellular proteins; however, some of the newly appearing bands could have been MT or HSP as well. Many xenobiotic compounds can bind to proteins, thereby possibly altering electrophoretic mobility (62). Hemoglobin levels were decreased in striped bass (*Morone saxatilis*) exposed to 5 and 10 ppb Mg and was due to a lower number of red blood cells rather than a decrease per cell (33). Many additional cases concerning pollutant effects on fish hematology were discussed by Heath (65,66).

O. mykiss exposed to the synthetic triaryl phosphate oil, IMOL-S-140, possessed decreased HB levels and greatly increased serum LDH and AAT activities (97). Asztalos and Nemcsók (5) found altered serum LDH electrophoretic patterns after treating carp (*Cyprinus carpio*) with paraquat, methidathion or $CuSO_4$; tissue damage (and resultant enzyme leakage) was responsible for the observed changes. Methidathion exposure of *C. carpio* resulted in a 77–92%

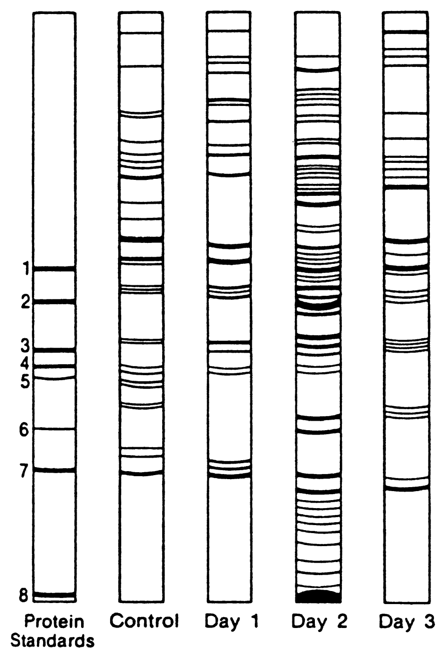

FIG. 1. Serum polypeptides resolved by SDS-PAGE from untreated (control) and MeHg treated (day 1–day 3) bluegills, *Lepomis macrochirus* (*n* = 4–5) (From Dutta, H.M.; Lall, S.B.; Haghighi, A.Z. Methyl mercury induced changes in the serum proteins of bluegills—*Lepomis macrochirus* (Teleostei). Ohio J. Sci. 83:119–122;1983 with permission.)

decrease in AChE activity and also resulted in the appearance of AChE molecular form G_1 (in both heart and muscle tissue), which was not detected in control fishes (168). Serum LDH, HBDH, AAT and ALAT were elevated in *O. mykiss* treated with formalin (200 ppm for 1 hr) or $CuSO_4$ (0.5 ppm for 1 hr), and after histological examination of livers, Wooten and Williams (198) concluded that the increased enzyme levels were the result of liver damage sustained from the two treatments. Bouck and Ball (20) suggested the possible use of LDH as a diagnostic tool for pollution-induced stress, in much the same way as LDH is used for detecting certain conditions in humans. SOD activity has been shown to increase in *Salmo trutta* due to increased ultraviolet light exposure (45) and in *C. carpio* due to paraquat and hypoxia (180). Increased quantities of SOD isozymes in *Zea mays* also resulted from paraquat treatment. Based on SOD levels after exposure to compounds that generate superoxide radicals and those that do not, it appears that the levels of superoxide radicals may control expression of *Sod* genes (146). Superoxide radical induces MnSOD in *Escherichia coli*, whereas the FeSOD is constitutive (64). The addition of paraquat also increased MnSOD synthesis. In a low Cu medium, synthesis of Cu/ZnSOD was suppressed and levels of MnSOD increased in the fungus, *Dactylium dendroides* (155). Increased synthesis of MnSOD, CAT and PER was observed in *E. coli* grown under aerobic vs anaerobic conditions (64). Exposure of bean plants (*Phaseolus vulgaris*) to ozone resulted in "expression" of two additional PER bands (107). Several detoxification and antioxidant enzymes displayed increased or decreased activities in *O. mykiss* treated with polychlorinated biphenyls compared with untreated controls (125). Di Giulio *et al.* (36) discussed the potential use of the antioxidant enzymes, SOD, CAT and GPX, as biomarkers of environmentally induced stress to aquatic organisms, and numerous studies on a variety of fish species were cited indicating increased activities of the three enzymes.

Racicot *et al.* (136) studied the effects of CCl_4 on plasma enzyme activities in *O. mykiss*. The first experiment involved the use of CCl_4 diluted in mineral oil with a subsequent 10-day monitoring period, whereas the second experiment monitored plasma enzyme levels for 24 hr after injection with pure CCl_4. Plasma enzyme levels generally increased soon after CCl_4 treatment due to liver damage (confirmed by histological examination); however, in both experiments, the enzyme levels fluctuated to varying degrees during the monitoring periods. In the first experiment, significant differences were found for LDH, AAT, ALAT, GDH and G6Pase for at least two of eight sample times. In the second experiment, activities of LDH, AAT, ALAT, GDH and GR were significantly greater at all times (6, 12, 18 and 24 hr) after CCl_4 treatment compared with controls. Experiments designed to determine xenobiotic effects on liver by monitoring blood enzyme changes could benefit from using test organisms with unusual tissue-specific expression of LDH-C such as some gadiform fishes (150, 152) and cyprinids (193) followed by electrophoresis of blood serum or plasma.

Hughes *et al.* (75) found that *Caradina* sp. exposed to an organophosphate insecticide experienced differential mortality dependent on the individual's GPI and PGM genotypes. Hughes *et al.* (75) cautioned however that "The possibility of the results reported in these studies being due to linkage (e.g., to an *Est* locus), rather than a direct effect on the enzyme being analyzed, cannot be discounted." This may be true, although selection appears to be occurring for one or more genes regardless. Certain EST allozymes in *Culex* sp. and *Musca domestica* confer resistance to organophosphate insecticides (131,172).

Gillespie and Guttman (54) found evidence that pollution was selecting for individuals of stoneroller (*Campostoma anomalum*) with certain PGM genotypes. PGM aa and bb genotype frequencies were significantly lower in *C. anomalum* from impacted sites vs upstream unimpacted and reference sites. A similar, but not statistically significant, relationship was observed for the frequency of MDH bb. Exposure of *C. anomalum* to Cu in the laboratory also revealed significant differences in sensitivity of PGM and MDH allozymes; PGM aa and MDH bb genotypes were more sensitive to Cu than PGM bb and MDH aa (54). Nevo (117) indicated that pollution can select for certain alleles by eliminating those individuals carrying the non-adaptive

allele(s). Nevo *et al.* (118) found significant differences in allele frequencies of *Balanus* sp. between a natural canal (lower temperature) and a canal receiving thermal pollution (9–12°C higher). Once a larval barnacle settles, it remains stationary; therefore, individuals with a particular genotype were either selectively eliminated or some form of isozyme replacement may have occurred (see 133). Betadine treatment of *O. mykiss* fertilized eggs appeared to select against individuals possessing the *Ck-A1*76* allele with a significantly lower frequency of this allele in the treated vs control group (93).

Exposure of caddisfly (*Nectopsyche albida*) to 0.6 mg/l $MgNO_3$ for 72 hr and subsequent electrophoretic analyses of survivors revealed significant differences for time to death among PGM, ADH-1 and ADH-2 genotypes (14). Significant correlations between genotype and time to death were also noted among genotype combinations at several loci (15). Chagnon and Guttman (30) found Cu exposure of eastern mosquitofish (*Gambusia holbrooki*) selectively eliminated individuals heterozygous at the *Gpi-2* locus with a significant difference between all heterozygotes and homozygotes and between *Gpi-2*ab* survivors and nonsurvivors of the Cu treatment. The *Idhp-2* locus in females was significantly affected with the aa genotype occurring in higher frequencies in survivors than nonsurvivors. Cadmium treatment also affected the bb genotype of *Gpi-2* with a significantly higher frequency of *Gpi-2*bb* in fishes surviving the treatment. Chagnon and Guttman (31) studied the effects of Cu and Cd on enzyme function via starch gel electrophoresis and subsequent staining with stains containing varying concentrations of the two metals. Different species exhibited differences in PGM-2 tolerance, and intraspecific differences were found in *G. holbrooki* PGM-2 allozymes, which differed in their tolerance to Cu. Gillespie and Guttman (55) found genotype frequencies of GPI-2 in spotfin shiners (*Cyprinella spiloptera*) to differ significantly among sites with varying degrees of water quality. After Cu exposure, time to death was significantly different among GPI allozyme genotypes of mayfly (*Stenonema femoratum*); thus, Cu exposure selectively eliminated carriers of certain GPI genotypes (13). Similar results for *G. holbrooki* and other organisms have also been reported (35 and references therein). In *Orchesella cincta*, Frati *et al.* (48) found a correlation between Cu tolerance and AAT alleles but did not find significant pollution effects on population gene frequencies.

Kopp *et al.* (87) consistently found significant differences in *Idhp* and *Pgdh* genotype frequencies in mudminnow (*Umbra limi*) between low pH/high Al^{3+} sites and reference sites within the Moose River, New York. Moderately consistent differences were also noted for *Gpi-1*, and *Mpi* and *Mdh-1*. A low pH environment may be lethal to fishes and is known to lower blood pH values by approximately 0.4 pH units between ambient pHs of 8.0 and 3.0 (127). Fishes exposed to low pH may be stressed due to losses of Na^+, Cl^- or K^+, and at times this stress can lead to death (187). Where acid precipitation is a problem, elevated levels of some metals are also likely (9,58); therefore, a combination of stressors would be present. HB content was increased in fishes chronically exposed to acid water (58), and the activities of several enzymes in Mozambique tilapia (*Oreochromis mossambicus*) were dependent upon previous pH conditions (16). Geographic distribution of certain general muscle protein patterns in four populations of a crayfish (*Cambarus bartoni*) appeared to be influenced more by the conductivity of stream water (or petroleum byproducts) than by geographic distance of the populations (173); however, further study is required to determine the nature of this variation.

Harmful compounds may also alter the long-term population genetic structure by increasing the number of mutant alleles and hence the level of polymorphism. For example, some offspring from a mouse previously exposed to the mutagenic compound *N*-ethyl-*N*-nitrosourea expressed five novel protein forms. Two proteins were identified as sEH and OAT, both of which differed in pI from other mouse sEH and OAT isozymes/allozymes (56,119). The physiological effects of toxins from algal blooms should be considered a potential effector of enzymes, especially in studies of marine molluscs. *Mytilus edulis* may retain toxins for periods ranging from 1 week to several months, and the toxins can affect feeding, growth and reproduction and cause mortality in many species of "shellfish" (156).

A systematic or population study of fishes that includes individuals from polluted sites may produce results that will be interpreted erroneously, possibly indicating that some populations are distinctive when, in fact, the observed gene frequencies are an artifact of exposure to environmental contamination. For example, one may conclude that population A possesses a unique allele for a protein because the allele was not detected in population B; thus, one may further deduce that gene flow is absent and divergence has occurred or some other such conclusion. Also, "Spatial distributions of allele frequencies do not themselves reveal how much gene flow is occurring." (159, p. 790). Suppose population B, or at least the locality from which the specimens were captured, was subjected to contamination that has killed, reduced or forced away fishes possessing the "unique" allele of population A. The apparent genetic difference may be found only on a very local scale. A study such as one by Howard and Morgan (74) in which allozyme variation was investigated in mottled sculpin (*Cottus bairdi*) across drainage divides could be affected by differential selection of genotypes from pollutant exposure or by temporarily decreasing or eliminating activity of some enzymes. Selection for particular alleles or genotypes could appropriately be called artificial selection. Potential sampling locations should be investigated for possible contamination from both point and non-point source pollution. Pollution exerts several effects on the physiology and biochemistry

of organisms and genetics of populations including altered synthesis of proteins/enzymes, changes in enzyme activity and changes in allele/genotype frequencies.

Indirect Effects

In addition to the direct effects pollution can have on a population's gene pool or an individual's enzyme complement or activities, there are a number of indirect effects as well. Fishes exposed to chronic levels of pollution may survive but may be more susceptible to bacterial or viral infections and parasite infestations (66 and references therein, 188), may have impaired feeding ability (66 and references therein, 189) and may experience reduced fecundity and survivorship (57,66 and references therein). The extent to which these may influence the results of electrophoresis is known in some cases (see Disease and Parasite sections).

State and federal agencies should be consulted concerning pollution impacts on specific streams, stream segments or other water bodies from which study organisms are to be collected. The Ohio Department of Natural Resources produces a yearly summary entitled *Water Pollution, Fish Kill, & Stream Litter Investigations* (e.g., 122). Also, the Ohio Environmental Protection Agency (Ohio EPA) has an extensive database on Ohio's stream fishes and macroinvertebrates (as well as water and sediment chemistry) that are used to assess the health of aquatic communities and determine sources of pollution (123,124). The Missouri Department of Conservation (MDC) produces a yearly report entitled, *Missouri Fish Kill and Water Pollution Investigations* (R.M. Duchrow, personal communication). Published reports by MDC cover investigations from the 1960s to present (38). Total numbers of fishes killed are listed in MDC reports but not species-specific data; detailed data can be requested, however (38). The United States Geological Survey and United States Environmental Protection Agency (USEPA) also have records of contaminant spills and fish kills. Lins *et al.* (96) provided 30 cases of pollutant spills nationwide, 16 of which resulted in fish kills of approximately 10,000–60,000 fishes; a natural fish kill was also listed, and a conservative estimate of the size of the kill was 100,000 fishes. USEPA (174) reported fish kills from 1960 to 1972 totaling nearly 300 million fishes and included 33 fish kills of >100,000 fishes in 1972 alone. Robison and Buchanan (142) provided a distribution map of Arkansas fish kills that occurred between 1961 and 1984 and cited several specific instances of fish kills in Arkansas and other states. Jenkins and Burkhead (78) discussed a massive fish kill that occurred in the Clinch River, Virginia and Tennessee. An estimated 216,000 fishes were killed, and this was considered a gross underestimate.

Holding all study organisms in captivity under controlled conditions should help eliminate variability in results. Holding time would allow for detoxification of contaminants before experimental regimens are initiated. Studies examining enzyme activities in which no consideration was given to the many effectors of activity may report false conclusions, and relationships that would otherwise have been apparent may be obscured.

PARASITES

Fishes as well as other vertebrates and invertebrates may be carrying parasites, and if parasites are homogenized with the tissue sample, some interesting problems may arise in gel interpretation (27,44,89). Vrijenhoek (181) encountered such variation in EST from eye tissue of a livebearer (*Poeciliopsis lucida*) some of the staining bands were due to a trematode infestation (neascus metacercariae). In a population study of the snail, *Bulinus senegalensis*, a variety of parasites contributed detectable GPI and MDH activity on gels (199). The distribution of parasites differed both geographically and seasonally among the seven *Bulinus* populations examined. Not accounting for the occurrence of parasites and the spatial and seasonal differences in parasites could lead to many false conclusions concerning differences in population genetic structure, gene flow, and so on. Significant seasonal and host age differences in abundance of gill parasites were reported for pumpkinseed (*Lepomis gibbosus*) and rock bass (*Ambloplites rupestris*) (60,61). GPI of the malaria parasite, *Plasmodium falciparum*, can be detected in host blood; the parasite was polymorphic at GPI, and the polymorphism was geographical (29). Meade and Harvey (105) studied the effects of infestations of a digenetic trematode (*Posthodiplostomum minimum*) on serum proteins of *Lepomis macrochirus*. Additional globulin bands resulted from the parasite infestation. Additional bands of GPI activity were detectable in bovine blood infected with *Theileria annulata* or *T. parva* (106). Using a bovine blood cell line, Dyer *et al.* (41) examined the effects of infection with *T. annulata* on phosphoprotein composition. *Theileria annulata* infected cells had three unique and two quantitatively increased phosphoproteins. The unique phosphoproteins could have been either parasite proteins or parasite-induced host proteins (41); however, one can see that comparisons of infected and noninfected blood without consideration of such would likely result in misinterpretation of the data. Stibbs and Seed (163) studied voles (*Microtus montanus*) infected with *Trypanosoma brucei gambiense* and found significantly higher hepatic and serum TAT in the infected voles.

Fish gill tissue is analyzed occasionally, and the glochidia of bivalve mollusks reside for a time in the gill tissue of a host fish. Research on how the presence of glochidia might affect electrophoretic analyses could be beneficial in two respects: we would know if they introduce variation into our electrophoretic studies and we may be able to identify a host fish for a species of bivalve if genetic markers are found for species of bivalves, thus elucidating the host/para-

site relationships that are of great interest and importance for maintaining diversity in bivalve populations (115,116,201). White *et al.* (190) reported preliminary studies using restriction fragment length polymorphisms to identify unionid glochidia from potential fish hosts. A thorough summary of data concerning fish hosts of bivalve mollusks was compiled by Watters (183).

The occurrence of parasites in fishes should be considered the rule rather than the exception. A glance at the percent incidence of infestation in publications on fish parasites supports the above statement (e.g., 7,10,34,67,144). Comparisons among individuals of a particular species, with some parasitized, could result in elevated levels of "polymorphisms." The same may also occur when individuals are infected by several parasites or when a species of parasite is found in all study specimens but is also polymorphic at some loci (e.g., 29,106). Tissue samples should be checked for any evidence of parasite infestations before homogenization (140, 181) or tissues can be homogenized and the remaining carcass can be examined for parasites later (105). Tissues used for electrophoretic studies are rarely examined for the presence of parasites. Whole body homogenates eliminate any chance of finding internal parasites, eliminate all tissues that could have been examined at a later time and allow proteins from ingested food to be detected. A solution for eliminating the parasite factor is to hold all fishes in captivity and administer treatments to all fishes and then use them several weeks later, allowing for a post-treatment recovery. Even when such treatments are used, tissues should be checked visually for parasites. These procedures will increase expenditures of time and possibly cost, if holding facilities are not available.

ADDITIONAL FACTORS

Food in the Gut and Bacteria

Enzymes from food in the gut of a study organism may also contribute variation (27,44). Some investigators have even conducted electrophoresis or IEF of partially digested remains from stomachs of invertebrates and vertebrates as a means of identification (63,82,113), indicating that some of the "foreign" enzymes retain detectable activity. Bacteria can also contribute their enzymes to extracts, resulting in patterns which may be either uninterpretable or misinterpreted (2,44). Gibson and Cavill (50) found *Paramecium aurelia* possessed an additional EST when growing in axenic media as compared with media containing bacteria. Three bacteria were tested, and all effected the EST loss from paramecia. The effect occurs approximately 6 days after exposure to bacteria. The bacteria must be live, but direct contact was not requisite. Gibson and Cavill (50) indicated that the responsible bacterial agent was proteinaceous. The *P. aurelia* EST reappeared after removal of the bacteria. Neuraminidase production by bacteria may also change the existing enzymes by cleaving sialic acids. A more thorough discussion of sialic acids and neuraminidase can be found in ref. 134.

Capture and Handling Stress

Capturing fishes via electrofishing may cause significant damage to tissues and initiate a biochemical response resulting in altered enzyme activities or gel patterns. Electrofishing could induce a heat shock or stress response because wounding is a known inducer of HSP (120). Wounding in plants induces expression of novel PER isozymes (43,90), and cycloheximide prevented the appearance of the new PER isozymes (90). Such variation would be most troublesome in a study using fishes captured by several methods (e.g., electrofishing and seining). Electroshocking fishes with either AC or DC current can cause fractured vertebrae and hemorrhage [(153) and references therein; (160,161,166); personal observation]. A damaged tissue (indicated by hemorrhage) may be leaking its tissue-specific isozymes into the bloodstream; enzymes are released in large enough quantities to be detectable by electrophoresis (refer to Disease and Pollution sections for examples). Electroshocked *O. mykiss* possessed elevated levels of CK in plasma compared with three other capture methods, whereas LDH did not differ significantly among the treatments (21). Bouck and Ball (19) investigated the effects of capture methods on HB and plasma proteins in *O. mykiss* and found minor quantitative differences among shocked, hooked and seined fishes. However, Bouck and Ball (19) used tricaine (MS-222; tricaine methanesulfonate), which can induce stress in fishes (186).

Hyperlacticemia and acidosis in muscle or blood may result from capturing fishes in the field or from attempts at capturing fishes from tanks if they are not netted quickly and are chased around the tank repeatedly. Lactate acidosis can cause death (28b). The rapid decline in blood/tissue pH can denature such isozymes as CK (184). Heisler (68) has diagrammed nicely the rapid drop and recovery phase of pH levels in blood and muscle of fishes (Fig. 2; see also Fig. 8.3 in ref. 68). Electroshocking caused a significant increase in blood lactate levels in largemouth bass (*Micropterus salmoides*) at 1 hr after capture, but by 3 hr post-capture, the levels had returned to normal (25). Even after organisms have been exposed to proper laboratory conditions for a suitable period of time, subsequent handling may induce stress. Because netting, handling or chasing may induce stress, it would be advantageous to devise alternative procedures to minimize any stress to study organisms. Marinsky *et al.* (103) transferred groups of *O. mykiss* from tank to tank without individual handling or air exposure by using cages that slip into tanks and that also hold enough water to keep fishes covered during transfers. Another way to avoid a stress response would be to apply a lethal dose of tricaine (>200 mg/l) to the tank from which the specimen is required. This procedure would necessitate holding one or

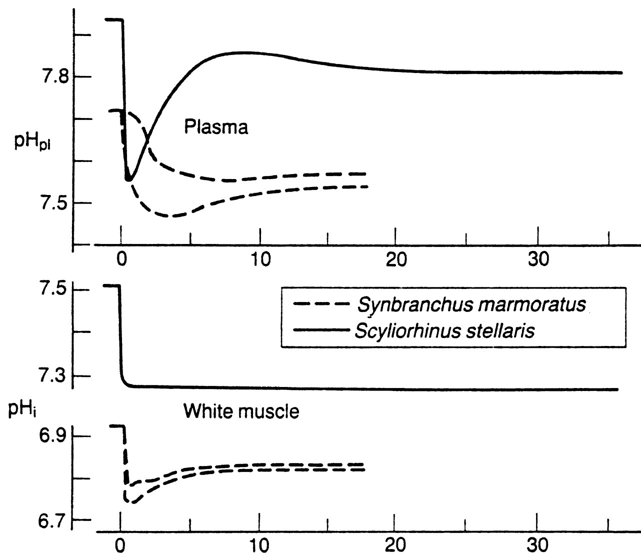

FIG. 2. Rapid decline in blood and white muscle pH in two fishes upon a 10°C increase in water temperature. The pH rises to a higher (although not the original; see Fig. 11-11 on p. 393 in ref. 71) value within several hours as a result of bicarbonate ion transfer. X axis is time (hr). (Reprinted from Heisler, N. Acid-base regulation in response to changes of the environment: characteristics and capacity. In: Rankin, J.C.; Jensen, F.B. (eds). Fish ecophysiology. London: Chapman & Hall; 1993:207–230, with permission from Chapman & Hall, London.)

only a small group per tank because tricaine obviously would not be selective. Neutralized tricaine does not initiate as strong a stress response as does unneutralized tricaine (186). Although the lethal dose of tricaine does not initiate a stress response (187), tricaine can affect the activity of some enzymes. Tricaine decreased the activities of aryl hydrocarbon hydrolase, EH and UDP-glucuronosyltransferase in liver of splake trout (91). Watts (184) recommended culling by physical means if possible to avoid any chemical effects on cellular membranes or enzymes. Houston and colleagues routinely use physical culling for just such reasons (88).

CONCLUSION

Many potential effectors of enzyme type, quantity or activity have been reported. While holding study organisms in captivity before initiation of experiments, one can observe individuals that may be diseased and either treat or eliminate those individuals. During the holding period, xenobiotic compounds can be eliminated from specimens and treatments can be administered for parasites. Also, holding study organisms in captivity allows for acclimation of standard environmental parameters such as temperature, photoperiod, pH, diet and so on that may affect enzyme activity and qualitative or quantitative expression [see (133)]. Controlling as many variables as possible may reduce nongenetic variation and other confounding variation and provide more accurate data from electrophoresis and enzyme assays.

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