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I am submitting herewith a dissertation written by Christopher W. Theodorakis entitled "Interpretation of Toxicological Data in an Ecological Setting: Physiological, Molecular and Population Genetic Responses of Fish to Genotoxicants." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

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# INTERPRETATION OF TOXICOLOGICAL DATA IN AN ECOLOGICAL SETTING: PHYSIOLOGICAL, MOLECULAR, AND POPULATION GENETIC EFFECTS OF GENOTOXICANTS ON FISH

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Christopher William Theodorakis
May 1994

## DEDICATION

To my family and friends, without whose love and support this would not have been possible.

**ABSTRACT**

In order to interpret genetic toxicological data in an ecological setting, studies were conducted to determine the relationship between laboratory and field research, and between individual and population-level responses. These studies are as follows:

1) The temporal expression of various genotoxic responses was determined in bluegill sunfish (Lepomis macrochirus) exposed under controlled laboratory conditions to sediment contaminated with genotoxic compounds (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and heavy metals). In addition, non-genotoxic responses were also examined over a chronic (up to 40 weeks) exposure. Genotoxic responses included DNA strand breakage, DNA adduct formation, cell-to-cell variation in DNA content (determined by flow cytometry), and changes in chromosomal protein profiles. Nongenotoxic responses included mixed function oxidase, acetylcholinesterase, and $Na^+/K^+$ ATPase activities, histopathology, stress protein induction, and hematological parameters. Biomarkers in the laboratory-exposed fish were similar to those of indigenous sunfish sampled from the site of origin of the contaminated sediment. Several patterns of development of biomarkers over time were evident, depending upon specific biomarkers and the tissue in which they were

examined.

2) DNA, isolated from the blood cells of the same bluegill sunfish as above, was examined for strand breakage by agarose gel electrophoresis under either neutral (pH 7) or alkaline (pH 12) conditions. The median molecular length (MML) of the DNA distributed in the gel was determined. These quantitative measures were used to estimate the difference in the number of double strand and single breaks between DNA preparations. Both types of strand breakage were found to be greater in fish exposed to sediment contaminated with genotoxic compounds as compared to non-exposed fish.

3) Mosquitofish (Gambusia affinis) were collected from Pond 3513, a small pond that is contaminated with $^{137}Cs$, $^{90}Sr$ and other radionuclides, from White Oak Lake, a settling basin contaminated with radionuclides and chemical genotoxins, and from non-contaminated populations. DNA was isolated from both liver and blood cells and examined for DNA total- (both single and double) and double-strand breaks alone by gel electrophoresis. In general, total- and double-strand DNA breaks were slightly more prevalent in fish from radionuclide-contaminated sites than from uncontaminated sites. In addition, there were differences in the number of strand breaks between liver and blood cells. Also, fecundity and number of malformed embryos were determined in fish from all

sites. It was found that, for fish from the contaminated sites, the number of DNA strand breaks was negatively correlated with fecundity, and that females with malformed embryos in their broods had slightly more DNA strand breaks than did females with no malformed embryos.

4) DNA polymorphisms, using the RAPD (Randomly Amplified Polymorphic DNA) technique, and allozyme polymorphisms were examined in mosquitofish (Gambusia affinis) from the same populations as above. The RAPD technique uses the polymerase chain reaction with a short oligonucleotide primer to produce DNA fragments which form banding patterns similar to DNA fingerprints. Using the RAPD technique, the genetic diversity of the contaminated populations were found to be greater than the non-contaminated populations. Furthermore, heterozygosity, both at the nucleoside phosphorylase locus and at all other loci, was found to be elevated in contaminated populations relative to control sites. Additionally, RAPD bands found at high frequency in Pond 3513 and White Oak Lake populations were present at a lower frequency or absent in the non-contaminated populations. For some primers, the contaminated populations showed more DNA bands per individual than the non-contaminated populations.

5) DNA polymorphisms revealed by RAPD and allozyme analysis were compared to relative amounts of strand breakage as

determined by gel electrophoresis in mosquitofish (Gambusia affinis). These fish were exposed to radionuclide contamination in the field and to X-rays in the laboratory. It was found in a Part 5 that in some instances the average number of bands and the frequency of certain bands were elevated in radionuclide-contaminated relative to reference populations. Therefore the median molecular length (MML) in each individual was compared to RAPD genotype in terms of the two metrics mentioned above. In several cases, the median molecular length (MML) of the DNA (which is inversely proportional to the amount of strand breakage) was correlated to the number of bands per individual. Also, for bands which had a higher population frequency in contaminated sites, the MML in many cases was higher for fish with than without these bands. Results from laboratory X-ray exposures paralleled those from the field.

The results of these studies will be discussed in terms of application to ecotoxicology and ecological risk assessment.

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## ABBREVIATIONS

AchE - acetylcholinesterase

ADA - adenosine deaminase

ALAD - δ-aminolevulenic acid dehydratase

ATP - adenosine triphosphate

BaP - benzo[a]pyrene

BaPDE - benzo[a]pyrene diol-epoxide

dATP - deoxyadenosine triphosphate

DCB - dichlorobiphenyl

dCTP - deoxycytadine triphosphate

DDW - double distilled water

dGTP - deoxyguanidine triphosphate

dTTP - deoxythymidine triphosphate

EDTA - ethylene diamine tetraacetic acid

EFPC - East Fork Poplar Creek

EROD - ethoxyresorufin-o-deethylase

FPS - Fish Physiological Saline

GPD - glucose-6-phosphate dehydrogenase

FUM - fumarase

Hb - hemoglobin

HBDH - hydroxybutarate dehydrogenase

Hct - hematocrit

ICD - isocitrate dehydrogenase

kb - kilobase

kDa - kiloDalton

LDH - lactate dehydrogenase

MDH - malate dehydrogenase

NP - nucleoside phosphorylase

PAH - polycyclic aromatic hydrocarbon

PAS - periodic acid Schift

PCB - polychlorinated biphenyl

PCR - polymerase chain reaction

PGM - phosphoglucomutase

PEP - peptidase

RAPD - randomly amplified polymorphic DNA

RFLP - restriction fragment length polymorphisms

RNase - ribonuclease

RNSB - relative number of strand breaks

SDS - sodium dodecyl sulfate

TBE - Tris-Borate-EDTA

TCB - Trichlorobiphenyl

TE - Tris-EDTA

TtCB - tetrachlorobiphenyl

UV - ultraviolet

ZPP - zinc protoporphyrin.

# PART 1

# INTRODUCTION

## OVERVIEW

The primary focus of this dissertation will deal with the interpretation of toxicological data in an ecological setting. This involves both examination of the relationship between laboratory and field studies and between individual and population-level responses.

The linkage between field and laboratory studies is important for two reasons. First, much of the toxicological data is gathered in the laboratory. This is essential for testing for unknown effects of various compounds. Also, the environment in the laboratory can be controlled and manipulated. However, in order to be useful for environmental risk analysis, effects in the laboratory must be applicable in an ecological setting. Therefore any results in the laboratory must be analogous to those found in field surveys. Second, if an effect is seen in the field, the assignment of a xenobiotic origin as the cause requires verification that the contamination in question causes this effect and elimination of extraneous environmental variables as the cause. Both of these objectives can readily be accomplished in the laboratory. In other words, by establishing a laboratory-field relationship, these two types of studies confirm and validate each other. Further, a such linkage between laboratory and field studies can be a first step in linking individual and population level effects; laboratory studies focus on individuals, while field studies often focus

on the population-level or above.

The linkage between individual and population-level effects is important for establishing that toxicological effects are of ecological relevance. If an effect is seen at the population level it could have the potential to affect community and ecosystem level responses. The importance of population vs. individual effects has ramifications to human society as well. The fate of individual organisms has little impact on society, unlike the fate of populations. In other words, the relationship between human society and the natural environment is dependant on population, rather than individual, effects. Therefore, the extrapolation of individual to population-level responses is of major importance for monitoring potential impacts of pollutants on both natural and human communities.

In order to elucidate these laboratory-field and individual-population relationships, this dissertation will primarily focus on genotoxins. This type of contamination was chosen because it is a major component of environmental contamination and is of major concern to both ecosystem integrity and human health. Genotoxins are chemical or physical agents which alter the structure and function of DNA in some detrimental way. These types of genotoxins include alkylating agents, organic chemicals which form covalent bonds with nucleotide bases (Lawley 1979); free radicals, which can cause strand breaks or form covalent bonds with nucleotide

bases (Schulte-Frohlinde and Vonn Sonntag 1985), and are formed from interactions of ionizing radiation with matter (Schulte-Frohlinde and Vonn Sonntag 1985) or from metabolic processes (Winston and DiGuilio 1991); ultra-violet radiation, which forms thymine dimers (Freeman and Thompson 1990); and heavy metals, which may react with DNA directly (Williams and Weisburger 1986), inhibit DNA repair enzymes (Hansen and Stern 1984), or contribute to the formation of free radicals (Winston and DiGuilio 1991).

A major focus of this dissertation in examining the effects of genotoxins on fish is the use of biomarkers. Biomarkers are molecular, physiological or anatomical aberrations that indicate organisms are being exposed to xenobiotic compounds and that these compounds may be producing an effect. The use of biomarkers in biological monitoring has several advantages. One is that there are a myriad of environmental variables which can affect the uptake, bioavailability, metabolism, and toxicity of xenobiotic compounds. Thus, one may not be able to assess if organisms are being affected by environmental contamination simply by monitoring contaminant concentrations in the environment. The second reason biomarkers could be important is that environmental contaminants are present as complex mixtures. Because monitoring for every possible contaminant may be prohibitive in terms of practical or economic limitations, it may be more feasible to monitor biomarkers in organisms living

in the affected ecosystems in order to see if contamination exposure produces any effect. Finally, if there is an ecological effect biomarkers may be useful in assigning this affect to some source of environmental contamination (Suter 1990). There are many biomarkers which could indicate exposure to genotoxic compounds or their effects, but this dissertation will focus mainly on DNA strand breakage.

In order to examine such genotoxic effects this study will be divided into five parts. The Part 1 deals with exposure of genotoxic sediment to fish in the laboratory. Originally this study was to focus primarily on genotoxic responses, but it was later expanded to include many non-genotoxic responses for a number of reasons. The first is that genotoxic compounds may have effects other than DNA damage. The second reason in that genotoxic compounds are usually present in the environment in complex mixtures that include genotoxic as well as non-genotoxic chemicals. Therefore, in order to properly assess the effects of genotoxic chemicals on aquatic organisms, such non-genotoxic endpoints also need to be addressed. Furthermore, Part 1 introduces several biomarkers or approaches to studying biomarkers which are not widely used in environmental biomonitoring. One such approach is the use of agarose gel electrophoresis to study DNA strand breakage.

The Parts 3 and 4 deal specifically with measurement of DNA strand breakage using agarose gel electrophoresis. This

method has been widely used in other areas of biomedical research, but has received little attention in environmental monitoring. The advantages for using such a technique are described in Part 3. A significant aspect of Part 3 is that it measures genotoxic resposes in the blood, a tissue which typically is not examined for genotoxic responses. The advantages to using blood as opposed to other tissues are discussed in Part 3. Blood DNA is also used in Part 4 and compared with results obtained using liver DNA (an organ more typically used for genotoxicity assays). This Part utilized the methods developed in Part 3 in a field situation. Furthermore, Part 3 examines the relationship between strand breakage and fecundity, which could be an important link between biomarkers and ecological effects.

In the Parts 2, 3 and 4, it was found that there could be a large amount of variability in response of organisms to genotoxic stress, and in Part 4 it was found that fecundity was correlated with the degree of strand breakage. These findings suggested that variation in response could have a genetic basis, and that certain individuals may have an adaptive advantage over others in the population. Therefore a study was undertaken to determine if exposure to genotoxic compounds (radionuclides, in this case) could alter population genetic structure, and if the magnitude of biomarker response (i.e., DNA strand breaks) could be correlated with genotype. The results of these studies are presented in Parts 5 and 6.

Part 7 discusses the results of this dissertation in terms of environmental monitoring and risk assessment.

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# PART 2

# SEQUENTIAL EXPRESSION OF BIOMARKERS IN BLUEGILL SUNFISH EXPOSED TO CONTAMINATED SEDIMENT

**ABSTRACT**

The temporal expression of various biological responses was determined in bluegill sunfish (Lepomis macrochirus) exposed under controlled laboratory conditions to sediment containing high concentrations of polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals. Liver, gill, blood, kidney, brain, spleen and intestine were removed from sunfish sampled at 1, 2, 4, 8, 16, and 40 weeks post-exposure. Biomarker data were recorded for specific proteins, enzymatic activities, DNA integrity, and histopathology. Biomarkers in the laboratory-exposed fish were similar to those of indigenous sunfish sampled from the site of origin of the contaminated sediment. Several patterns of development of biomarkers over time were also evident. For example the responses of certain biomarkers are not time-dependant (i.e., intestine and gill ATPase activities), while that of others, such as brain ATPase activity, liver cytochrome $P_{450}$ and NADPH content, stress proteins, chromatin proteins and DNA strand breaks, fluctuate over time. Still other biomarkers, such as EROD activity, zinc protoporphyrin content of the blood, and DNA adducts, showed marked increases over time. Such patterns need to be considered when comparing laboratory and field results and deciding which biomarkers to use for biomonitoring programs. Implications for natural

selection and population/community level responses are also discussed.

# INTRODUCTION

There is a need for more work demonstrating that biological responses to pollutants in the laboratory are comparable to field data. This need arises for several reasons. First, most studies that examine the effects of anthropogenic contaminants on aquatic organisms focus either on laboratory or field research, with only a few investigations directly linking the two. Second, a large proportion of laboratory studies report results obtained from a single time point or from short term exposures with relatively little information on long term development of biological aberrations in response to toxicants. More detailed information is needed to understand the mechanisms of action of pollutants on aquatic animals, and is necessary to properly interpret laboratory findings with respect to field data and vice versa. Results obtained in the lab may be contradictory to field data if the improper length of exposure is chosen (e.g., Shugart 1988). Third, in the field it is difficult to control environmental variables which may affect or modulate toxicological responses. Therefore field data must be validated with appropriate laboratory studies in which these environmental correlates can be controlled.

Many studies, both in the laboratory and in the field, focus on only one or, at most, a few endpoints. Although focusing on a small number of endpoints may be adequate for

monitoring exposure to a particular compound, it has limited utility in predicting the effect of pollution, that often occurs as a complex mixture in the environment, on the overall health of the organism. Therefore there is a need for more studies examining multiple biological endpoints in response to toxic stress. Currently there is a trend in ecotoxicology to use toxic responses of organisms as sentinels of environmental contamination. These biological indicators of contamination (referred to as "biomarkers") can be much more sensitive and ecologically relevant than simply measuring the levels of contaminants in the environment (McCarthy and Shugart 1990). Thus, information from studies with multiple endpoints would also be useful in determining which organ systems or tissues would be the most sensitive or relevant systems to obtain biomarkers data. There is also an effort underway to develop short-term (e.g., biochemical and molecular) predictors of long-term (eg., population and community) effects on aquatic systems (Adams et al. 1989). In particular, the Biological Monitoring and Abatement Program (Loar et al. 1988) at the Oak Ridge National Laboratory has been monitoring the biological responses of aquatic organisms in East Fork Poplar Creek (EFPC). EFPC receives industrial effluent from the Y-12 Plant, a U.S. Department of Energy (DOE) nuclear weapons production facility. Redbreast sunfish (Lepomis auritus) in EFPC have been found to have elevated tissue levels of PCBs and mercury (Kornegay 1991), as well as biomarkers indicative

of contaminant-induced stress (Adams et al. 1990, Shugart 1990, Adams et al. 1989). These biomarkers have also been correlated with community and ecosystem level responses, such as species diversity and community structure (Adams et al. 1989).

Therefore the principal aims of this study are: 1) to examine the sequential and temporal development of various biomarkers in a fish exposed to a known contaminated sediment over a reasonable period of time; 2) to evaluate and compare a wide suite of biomarkers in a variety of organs and organ systems, and 3) to determine if biological responses observed in the laboratory-exposed fish are comparable to those seen in the field. These objectives were addressed by exposing bluegill sunfish (Lepomis macrochirus) in the laboratory to EFPC sediment for various periods of time. Endpoints include a suite of bimolecular, anatomical and histological responses. Particular endpoints were chosen because either they are altered: 1) in redbreast sunfish in EFPC; 2) in fish or other organisms from areas with similar pollution; or, 3) in fish exposed in the laboratory to compounds known to present in the sediment of EFPC (Table 1). The particular organs examined were chosen because they were primary target organs or were the site of exposure and/or detoxification.

# METHODS AND MATERIALS

## Chemicals

All reagents and enzymes explicitly mentioned in this paper were purchased from Sigma Chemical Co. (St. Louis, Mo), unless otherwise stated. Where the reader is referred to another paper for specific methodology, the sources of the reagents used in those assays can be found in their respective references. All organic solvents were purchased from J.T. Baker (Phillipsburg, NJ).

## Sediment Source

Contaminated sediment was collected by grab sample near the headwaters of EFPC, just downstream from the Y-12 plant. Control sediment was collected from a relatively clean headwater stream on the DOE Reservation.

## Experimental Design

The collected sediment was sieved through a 2 mm metal sieve. Approximately 3 L of wet sediment was placed in the bottom of each of 12, 76-L glass aquaria. Six of the aquaria received contaminated sediment and 6 received control sediment. Thirty-two L of charcoal-filtered, UV sterilized,

dechlorinated tap water was then added to the aquaria and allowed to equilibrate with the sediment for one week before fish were added. In addition, approximately 30 L of contaminated sediment was added to a 378-L aquarium. This aquarium was filled as needed with water in order to provide fresh, contaminated water to the contaminated tanks. The water was continuously aerated throughout the experiment in all aquaria. Ten hatchery-reared bluegill sunfish, measuring 80-100 mm (standard length) and weighing 25-40 g, were then added to each aquarium (5 males and 5 females). The fish were acclimated to laboratory conditions in clean water for one week prior to the start of the experiments, and fed ad libitum 4 times per week with approximately 17 g of live tubificid worms (Tubifex spp.) apportioned to each tank at each feeding throughout the experiment. Fish in the contaminated and control tanks were fed worms which were kept for one week in the contaminated and control sediment, respectively. Every day approximately 1/4 of the water from each tank was removed and replaced with fresh contaminated or uncontaminated water, depending on the treatment. Water from the 378-L aquarium was allowed to equilibrate for at least 3 days with the contaminated sediment before being used to fill the contaminated aquaria.

Fish were sampled from the tanks at 1, 2, 4, 8, 16, and 40 weeks after the exposure was begun. Although there were 6 aquaria total for each treatment, only 3 of the aquaria were

sampled at each sampling period, with the other half being sampled at the next sampling period. In order to minimize tank effects, the 6 control and 6 exposed tanks were each randomly designated to be sampled at week 1 or week 2 (3 tanks total for each week for each treatment). This randomization procedure was repeated for weeks 4 and 8 and then for weeks 16 and 40. Although both male and female fish were exposed to the sediment, only the analysis for females are reported here. All fish were anesthetized in a solution of 50 mL dimethyl ether/L water, and blood was collected in EDTA or heparinized vacuum tubes *via* caudal vein puncture. The fish were then sacrificed by overdose of anesthetic followed by spinal scission. Organ samples were frozen in liquid nitrogen and stored under liquid nitrogen or until analysis. Samples of skeletal muscle were frozen for PCB and heavy metal analysis. Organ tissues were not used for chemical analyses because they were used in biomarker analyses.

**Sediment Analysis**

Both control and exposed sediments were analyzed for heavy metals (EPA method 200.7, U.S. EPA 1983), hexane-extracted (EPA method 3550, U.S. EPA 1986) and analyzed for PCBs and PAHs (EPA methods 8080 and 8270, respectively, U.S. EPA 1986). PCBs were quantified by Aroclor number (EPA method 8081, U.S. EPA 1986) and by congeners. Individual congeners

were identified according to retention time, and the external standard method using peak area for quantification. Eleven dichloro- to hexachloro-biphenyl congeners were identified. Congener standards were obtained from ULTRA Scientific (Hope, RI).

**Water Analysis**

Physical and chemical parameters - At the time of sampling of the fish, water samples were also collected, filtered through a 0.45 $\mu$m filter and centrifuged at 10,000 rpm for 15 minutes to remove suspended particles. The temperature and pH were measured immediately after collection. The pH was measured on an Orion pH meter (Orion Research, Inc.). The remainder of the water was acidified with Ultrex nitric acid (J.T. Baker, Phillipsburg, N.J., 1 ml acid/L water), and stored at 4° C until analysis (no more than 24 hrs storage for organic extraction). The amount of organic carbon dissolved in the water was also monitored throughout the experiment. Total dissolved organic and inorganic carbon was determined on an O.I. model 700 total organic carbon analyzer (O.I. Corporation, College Station, TX). Metal concentrations in the water were determined by inductively coupled plasma (ICP) spectroscopy (EPA method 200.7; U.S. EPA, 1983).

PCB analysis - Fifty ml of water were extracted as described by Dillon and Burton (1991), the extracts were dried and the residue was resuspended in 1 Ml hexane. All glassware was acetone-rinsed before use. One $\mu$L hexane extract was injected into a Perkin-Elmer model 8500 gas chromatograph equipped with a DB5 fused-silica capillary column (30 m x 0.25 mm I.D.) and an electron capture detector. Temperature programming followed established EPA procedures (EPA method 8080; U.S. EPA 1986). Congeners were identified as above.

PAH analysis - Ten mL of water were passed through a C-18 Sep Pak cartridge (Waters Associates, Milford, MA). The PAHs were then eluted with 3 mL methanol, concentrated to 100 $\mu$L, dissolved in acetonitrile/water (1:1) and analyzed by HPLC with fluorescence detection (Maccubbin et al. 1984). Quantitation was performed as with the PCBs. PAH standards were obtained from ULTRA Scientific (Hope, RI).

**Analysis of Biological Materials**

PCB analysis - Fillets were ground with $Na_2SO_4$ and Soxhlet extracted (EPA method number 3540; U.S. EPA 1986) with acetone as the solvent. The extract was concentrated on a rotary evaporator, and the residue was redissolved in hexane with 3, 3-mL washes of the evaporator flask. The extract was taken to dryness and redissolved in 5 mL hexane. A 1 mL aliquot was

removed for lipid determination by gravimetric methods. The remainder was cleaned on a Florosil column and analyzed by gas chromatography as before. PCB's were again identified according to congener and Aroclor number.

Heavy metal analysis - Fillets were ashed (Stahr et al. 1977), dissolved in 1N HCl, filtered, and diluted to 10 mL with double distilled water. Metal determinations were performed on an ICP spectrophotometer as with the water. All glass- and porcelainware was acid washed and DDW rinsed before use. The same preparation for metal and organic chemical analyses were performed on the tubificid worms after 1 week of exposure to the contaminated sediment, with the exception that the PAHs and PCBs were extracted with sonication (EPA method 3550; U.S. EPA 1986).

**Biomarker Analyses**

Protein determinations - Protein concentrations were determined for all assays using the method of Bradford (1976) with Bio-Rad reagents (Bio-Rad Corporation, Richmond, VA).

$Na^+/K^+$ ATPase activity - Frozen brain, gill and kidneys were homogenized with a glass homogenizer in a buffer consisting of 300 mM sucrose, 20 mM EDTA, 100 mM imidazole and 0.1% sodium deoxycholate (Zaugg 1982). ATPase activity of the

homogenate was measured as the cleavage of ATP to form inorganic phosphate. Inorganic phosphate was measured spectrophotometrically (Ames and Dubin 1960) before and after incubation of tissues at 37° C for 10 min. Appearance of additional phosphate was assumed to be attributable to ATPase activity. $Na^+/K^+$ ATPase was discriminated from all other ATPase or phosphorylase activities by comparing the results of the assay with and without ouabain (Zaugg 1982).

Acetylcholinesterase activity - Brains were homogenized with 0.25-0.5 mL of 150 mM NaCl, 6 mM Tris buffer (pH 7.4), 1 mM $MgCl_2$, and 0.5 mM EDTA. Then 0.1 mL of this homogenate was mixed with 0.45 mL of $Na^+$-$K^+$ phosphate buffer, pH 8.0, and the activity was determined spectrophotometrically (Halbrook et al. 1991).

Liver and spleen parameters - Liver microsomes were prepared by homogenizing tissues in sodium phosphate buffer using a motor-driven Poter-Elvehjem glass and Teflon homogenizer (Talboy's Instrument Co., Emerson, N.J.; Jimenez et al. 1988), and subsequently isolating the microsomes by differential centrifugation (McKee et al. 1983). Liver microsomal ethoxyresorufin-O-deethylase (EROD) activity was determined fluorometrically by an automated procedure, while cytochromes $P_{450}$ and $b_5$ concentrations were determined spectrophotometrically (Jimenez et al. 1988). Microsomal

NADPH cytochrome-c reductase activity was measured photometrically by the methods of Phillips and Langdon (1962), with a modified reaction mixture consisting of 50 mM Tris (pH 7.43), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1.1 mg/ml horse heart cytochrome c, 0.175 mM NADPH, and 2-10 $\mu$g microsomal protein (Loar et al. 1988). NADH-cytochrome $b_5$ reductase activity was measured by methods identical to that for NADPH cytochrome c reductase, except that 0.25 mM NADH was used in place of NADPH (Loar et al. 1988). Cytochromes $P_{450}$ and $b_5$ concentrations were each measured using their characteristic oxidized and reduced spectra (Johansen and DePierre 1978). Liver and spleen somatic indices were measured as a percent of total body mass.

Bile metabolites - The bile was diluted to 0.5% in DDW and analyzed by HPLC with relative quantitation using BaP as the standard (Krahn et al. 1986).

DNA damage - DNA strand breaks were determined by an alkaline unwinding assay and expressed as "F values" or "n values" (Shugart 1988). The F-value is inversely proportional to the number of strand breaks. The n-value is a measure of the relative amount of strand breaks in exposed fish compared to controls. For example, an n-value of 1.5 indicates that exposed fish have 150% more strand breaks than controls. In addition, an alternative method of determining relative strand

breaks was performed. This was done using acrylamide gel electrophoresis under alkaline conditions (Freeman and Thompson 1990) with the same DNA as the unwinding assays.

DNA adducts of BaP were determined for gill, liver, and blood DNA. DNA from livers and gills was isolated by homogenization in a buffer solution (250 mM NaCl, 100 mM tris-HCl, 100 mM EDTA and 1% Sarcosyl detergent), extraction with phenol/chloroform and chloroform, and digestion for one hour successively with RNase A and proteinase K (100 $\mu$g/mL for both) at 50° C. The digestate was extracted again with phenol/chloroform and chloroform. The DNA in the extract was precipitated with ethanol, dried and resuspended in 1 mL DDW. A 100 $\mu$l aliquot was removed for DNA determination by fluorescence methods (Shugart 1988). BaP adducts in the remaining portion were acid hydrolyzed and quantitated by HPLC with fluorescence detection (Shugart 1987).

Blood cell nuclei were isolated by the methods of Hewish and Burgoyne (1973), except that phosphate-buffered saline plus 10mM EDTA was used as the buffer. The cells were lysed with Nonidet P-40 detergent and the nuclei were collected with centrifugation. The nuclei were lysed with 1% Sarcosyl detergent and the DNA was purified and the adducts were quantitated as above.

Hemoglobin adducts - After the blood cells were lysed and the nuclei pelleted, the supernatant containing the hemoglobin

was collected. The globin was separated from the heme and precipitated in acidified acetone (Shugart 1985). The globin was then dried, weighed, and resuspended with sonication into 1 mL DDW. The BaP-globin adducts were quantitated as above.

DNA content and variability - Frozen liver samples were transported in liquid nitrogen to the Center for Biosystematics and Biodiversity at Texas A&M University, and stored at -63° C until analysis. Monodispersed nuclear suspensions for flow cytometry (FCM) were made from 2-3 mm blocks of the tissue samples. Tissue suspensions were prepared following the Detergent-Trypsin method for the preparation of nuclei for FCM analysis by Vindelov et al. (1983). The intercalative binding fluorochrome propidium iodide (PI) was used to form fluorescent complexes with DNA in the tissue suspensions. RNA in the suspensions was destroyed by pretreatment with Rnase. The stained tissue suspensions of both control and exposed samples were assigned numbers and blindly analyzed for relative DNA content on a Coulter EPICS Profile II flow cytometer. The Profile II was equipped with a 488 nm Argon laser to excite the DNA-PI fluorescent complexes.

Blood parameters - Delta($\delta$)-aminolevulinic acid dehydratase activity (ALAD) was determined colorometrically (Mitchell et al. 1977). Initially, EDTA and citrate, both

efficient chelators of metal ions (Klaasen et al. 1986), were used in the assay, but this may have affected enzyme activity. Therefore a second assay replaced EDTA and citric acid with heparin and HCl.

Zinc protoporphyrin (ZPP) content of fresh blood was measured on an automated zinc protoporphyrin meter (Aviv Corp., Lakewood, N.J.). Hematocrits were measured by drawing up fresh blood into a microcapillary tube and spinning it for 10 minutes in an EIC MB micro hematocrit centrifuge (Needham Heights, MA).

Histopathology - Once tissues were removed from the specimens, they were immediately immersed in Karnovski's fixative. Fixed tissues were embedded in paraffin following routine procedures, section at 5 $\mu$m, and stained with hematoxylin and eosin for general observations or with periodic acid-Schiff (PAS) reagent for demonstration of complex carbohydrates (Bancroft 1977). After staining, slides were numbered and shipped to the Gulf Coast Research Laboratory for blind analysis.

Stress and chromosomal protein analysis - For stress protein determinations, whole frozen tissues were shipped in liquid nitrogen to the University of Maryland (Baltimore County Campus) for analysis. Upon arrival, gill homogenates were prepared and electrophoresed on a polyacrylamide gel

according to established procedures (Bradley and Ward 1989). Gels were then stained with Coomassie blue (Higgins and Dahmus 1979) or Western blotted (Burnette 1981) with polyclonal anti-$HSP_{70}$, which crossreacts with $HSP_{90}$ in daphnids (Bradley 1992). The stress proteins were visualized with peroxidase-linked anti-IgG (Winston 1989).

For analysis of red blood cell chromatin proteins, blood cell nuclei were prepared from 50 $\mu$L of blood using the techniques of Hewish and Burgoyne (1977). The chromatin proteins were prepared and electrophoresed on a 13% polyacrylamide gel (Laemmli 1970) and stained as above. The relative amounts of proteins in each band were compared qualitatively using a Scanning Laser Densitometer (LKB Producter AB, Bromma, Sweden).

**Statistics**

Because the numerical distributions of many of the samples seemed skewed, differences between control and exposed fish were determined using a nonparametric (Wilcoxon rank-sum) test (Hollander and Wolfe 1973). The authors acknowledge that a rigorous statistical study of the data was not possible because of the small sample size (n = 3, unless otherwise noted), however, the experimental design was predicated by the logistical constraints on available laboratory space, personnel, and the long duration of the exposure. Moreover,

the objectives of this study are more concerned with qualitative trends with time of exposure rather than an estimation of precise quantitative differences between fish.

## RESULTS AND DISCUSSION

### Chemical Analysis

Physical and chemical parameters for water samples are listed in Table 2[1]. These values did not significantly differ between aquaria within treatments ($P > 0.50$), and were also similar between treatments (i.e., contaminated and uncontaminated aquaria). Organic carbon content of the water did not vary between aquaria or treatments or over time, except for the slightly elevated levels the first week (Table 2).

Determination of the concentrations of the various xenobiotics in the sediment, water and fish tissues was necessary in order to show a causal relationship between exposure and response. PCB analysis included both congener-specific and aroclor specific analyses. This was because previous studies determined PCB loads using Aroclor specific analyses. Significant levels of PCBs, PAHs and heavy metals were present in contaminated sediment (Table 3). No PAHs were detected in the control sediment. PAHs, PCBs and metals were also present in the water equilibrated with EFPC sediment

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[1]All tables and figures for Part 2 are found in the appendix to Part 2.

(Table 4). There were no differences within treatments ($P > 0.50$) and there were no consistent trends over time.

The worms used to feed the fish exposed to EFPC sediment had high levels of PAHs, PCBs and heavy metals (Table 3). These contaminants were not detected in the worms fed to control fish. Also, the stomach contents in a subsample ($n=12$) of fish fed just before sacrifice contained an average of 0.04 g worms/g body weight.

In the fillets, PCB concentrations were high (Table 5) but heavy metal concentrations were low (Table 6). While heavy metal concentrations fluctuated over time with no consistent trends (Table 6), PCBs showed a general trend of increasing concentration over time (Table 5). The lower chlorinated congeners had lower bioconcentration factors than did the higher chlorinated congeners (Table 5). The total PCB concentration, determined by Aroclor analysis, of week 16 exposed fish was 5.55 $\mu$g/g. Recovery of spiked samples ranged from 85-94% for various PCB congeners and 70-95 % for metals. Precision ranged from ± 5-8% for PCBs and ± 8-12% for heavy metals.

These analyses indicated that PAHs, PCBs and heavy metals were accumulated by the fish, giving credence to the idea that it was these chemicals that affected changes in biomarker responses. In addition, analyses also revealed congener specific patterns of PCB bioaccumulation which are similar to findings from the literature (See Shaw and Connell 1986).

These results provide strong evidence that the source of the PCBs is the sediment. Aroclor-specific analyses were included in this study to compare results with reported results from EFPC. However, we feel that Aroclor analyses may underestimate the actual levels of PCBs as reflected by the results of this study, especially for less chlorinated congeners. This may be due to the different congeners in pure industrial mixtures having different uptake, depuration, sediment adsorption/desorbption and degradation rates and different aqueous solubilities. Reported values of PCB body burdens for EFPC fish were much less than those in the present study. This may be due to the fact that congener-specific analysis was used in the lab but Aroclor-specific analysis was used for EFPC fish. However, even the subsample of laboratory-exposed fish in which the PCBs were identified as Aroclors were found to have higher concentrations than EFPC fish. This could be due to different exposure concentrations in the field than in the lab, or to the fact that a different species was used in the lab (bluegill) than in the field (redbreast sunfish). Differences in bioaccumulation or biomarker response between individuals from different tanks and time points were apparently not due to fluctuations in water concentrations of contaminants, which remained relatively constant. It is interesting to note that the heavy metals present seemed to equilibrate rapidly with the body tissues of the fish, while the PCB concentrations increased

over time. These contrasting patterns of accumulation may have had an effect on the temporal patterns of biomarker expression described below.

**Biomarker Analyses**

All probability (alpha) values refer to the Wilcoxon rank-sum test. Data for some weeks are missing due to insufficient tissue to perform all assays on all samples. Although all fish had well developed gonads, reproductive activity was not apparent during this study. The results will be discussed according to organ system and/or the trend of response.

Brain enzyme activity - Both AchE and brain $Na^+/K^+$ ATPase activities were higher in exposed fish relative to control after 1 week, but decreased over time (Fig. 1). Because the brain enzyme activities are affected by xenobiotics, there may also be implications for behavioral toxicology studies. Most behavioral studies involve short-term exposures, but the results from this study indicate that the effects on brain enzyme activities from long-term exposures are different from short-term exposures, and alterations of behavioral patterns may follow the same trends. In laboratory studies, short-term exposure to some xenobiotics produces behavioral hyperactivity in fish (Drummond and Russom 1990, Steele 1987), which is

consistent with increased enzyme activities in the present study. But the results from this study imply that long-term behavioral alterations might be different from the short-term.

Gill and intestine parameters - The $Na^+/K^+$ ATPase activities of the gill and intestine were decreased in exposed fish compared to controls (Fig. 2). Histopathological examination of the gills of exposed specimens indicated erosion of the epithelium and damage to the secondary lamellae. With PAS staining there appeared to be a reduced number of mucous cells in many exposed fish. None of the changes, however, showed temporal trends.

Liver parameters - In exposed fish EROD activity increased with time and was greater than controls (Fig. 3). The liver somatic indices seemed not to be affected until the $4^{th}$ week, but thereafter showed a trend similar to EROD. Microsomal concentrations of cytochromes $P_{450}$ and $b_5$ showed fluctuations in response but no consistent trends over time. The same was true for liver microsomal NADH-cytochrome $b_5$ and NADPH-cytochrome c reductase activities. In general, the concentrations in exposed fish for these parameters was higher than in controls, especially for the longer exposures (Fig. 4). Histopathological examination of liver tissue showed focal necrosis in specimens at 1 and 4 weeks of exposure. In several 8- and 16-week specimens, scattered individual

hepatocytes appeared necrotic. PAS staining indicated glycogen depletion in exposed specimens at 1 and 4 weeks.

Bile metabolites - The bile contained many fluorescent compounds found to chromatograph with retention times similar to PAH metabolites (Krahn et al. 1984). Relative amounts of these chemicals in the bile of exposed fish were found to be over 300-fold greater than those in control fish (Table 7), but did not vary with time.

DNA damage - At one week there were more strand breaks in the exposed than control fish. After two weeks there was no difference between treated and control fish, while after 8 weeks the exposed fish seemed to have fewer strand breaks than controls. After 40 weeks of exposure the contaminated fish again appeared to have more strand breaks than controls (Table 7). Short-term results in this study are comparable to other laboratory studies (Shugart 1988), but different from results from the field (Shugart 1990). Temporal fluctuations in the level of strand breakage could be due to changes in activity of DNA-damage compensatory mechanisms.

Results from the gel electrophoresis assay indicated that samples with lower F values (i.e, more strand breaks) had a greater electrophoretic mobility (i.e., lower molecular weight) than samples with higher F values (Fig. 5). This indicates that gel electrophoresis could be used as an

alternative method for examination of DNA strand breakage. Quantification could be accomplished with laser densitometry (Freeman and Thompson 1990). An advantage of this technique is that it requires much less tissue than the alkaline unwinding assay.

BaP-DNA adducts in the gill were apparent after the first week of exposure, with increasing concentration over time (Table 7). In contrast, adducts to liver DNA were only detected in the longest exposures. Blood DNA adducts were apparent after only one week and increased more rapidly than in the gill (Table 7). The chromatograms also suggested fluorescent adducts other than BaPDE were also present.

Adducts to the hemoglobin were detected after one week of exposure, increased after two weeks, then remained relatively constant over time (Table 7). No DNA or hemoglobin adducts were detected in control fish. Measurement of hemoglobin adducts, although less sensitive than DNA adducts, could be used as a surrogate to assess exposure to genotoxins. Other studies show a correlation between hemoglobin adducts, genotoxicity and carcinogenesis in mice (Mus musculus; Shugart 1986).

DNA content - DNA content, as determined by flow cytometry, of cells in the $G_1$ phase of the cell cycle exhibited a normal distribution about the mean. The relative DNA content for each fish was measured as the mean content for

$G_1$ cells, and cell-to-cell variation in DNA content was measured as the % coefficient of variation about this mean. The average amount of DNA was found to be greater in control than exposed liver cells, but this pattern is only significant at the 0.05 level at 8 and 40 weeks of exposure (Table 7). The cell-to-cell variation in DNA content was slightly greater in exposed than in control fish (Table 7). An exception to this pattern is the variation in DNA content in exposed fish at 8 weeks, which is less than that of control fish. There were no differences between control and exposed fish in the proportion of cells in G1, S or G2 phases of the cell cycle ($P>0.30$, data not shown). This indicates that flow cytometry can be used to detect genotoxic responses in fish. What is less clear, however is the molecular basis for these patterns. Because the relative proportions of cells in each phase of the cell cycle are the same in control and exposed fish, it is unlikely that these patterns are due to different rates of mitosis. Increased cellular variability, as a result of mutagen exposure, was observed in small mammals (McBee and Bickham 1988) and slider turtles (Trachemys scripta; Lamb et al. 1991, Bickham et al. 1988) exposed to environmental genotoxins and in laboratory rats exposed to the clastogenic drug triethylenemelamine (Bickham et al. 1992). Although the molecular basis for the alteration of mean DNA content is unclear, increased cell-to cell variability is likely due to chromosomal breakage (Bickham 1990, Bickham et al. 1992).

Blood, kidney and spleen - ALAD activity, when EDTA and citric acid were used in the assay, was greater in exposed than in control fish (Table 8). This pattern was opposite of what has been found in other studies of fish exposed environmentally to lead (Hodson et al. 1980). When EDTA and citrate were replaced with heparin and HCl, the activity of the exposed fish was found to be lower than that of control fish (Table 8). It should be noted that the control fish did not seem to be affected by the assay protocol. ALAD inhibition is due to non-covalent binding of the metal to the protein (Finnelli et al. 1975), so the presence of chelators in the assay may promote dissociation of metals from the ALAD molecules. If so, higher ALAD activity in exposed fish with chelators suggests higher concentrations of enzyme than in control fish, which may be a compensatory response to low enzyme activity. Further work is needed to clarify these results.

Hematocrits were higher in exposed fish after one week, but fell below controls thereafter (Fig. 6). One should be aware that hematocrit is also affected by physiological and reproductive states (Blaxhall 1972), thus field results should be interpreted with caution.

ZPP content of the blood was consistently higher in treated fish, with the values increasing over time (Fig. 6). It has been shown that zinc can substitute for iron in heme molecules if porphyrin synthesis is disturbed by lead (Cory-

Slechta et al. 1989; Labbe and Rettmer 1989) or other metals (Missenar et al. 1989; Flora et al. 1991). Thus an elevated level of ZPP in the blood can be used as a biomarker of pollutant exposure, but to our knowledge there are no studies that have used the levels of ZPP in fish blood as a biomarker. It has been found, however, that great blue herons (Ardea herodias) roosting along Poplar Creek have ZPP levels very similar to the exposed fish at week 8 (unpublished data). The trend of increasing response over time can be seen with the spleen somatic index, but the only significant difference between control and exposed fish was week 16 (Fig. 6). These markers indicate a general disruption in hematopoietic function.

Kidney ATPase activity in exposed fish was not depressed until week 16 (Fig. 6). Indications of renal damage, tubular degeneration and the presence of eosinophilic inclusion bodies in tubular epithelial cells, occurred in 2 of the 3 fish at week 16. No histopathological lesions were found in kidneys of control specimens from that time period. Thus tubular damage is indicated at both the tissue and biochemical levels after chronic exposure to toxicants.

Stress protein analysis - Western blots of $HSP_{70}$ from the gills indicated that this stress protein is present at higher concentrations in exposed fish at weeks 1 and 2. However, there was no difference in the amount of $HSP_{70}$ from weeks 4 and

8, and by week 16 the amount of this stress protein in exposed fish was less than that from controls. Total staining of gill proteins indicated that proteins of 55 and 90 kDa were expressed more strongly in exposed fish from 1,2,4,and 8 weeks of exposure. However, the 90 kDa protein did not cross-react with anti-$HSP_{70}$, as it does in Daphnids (Bradley 1992) and other species (Gonzalez and Bradley unpublished data). Both Western blot and total protein analysis revealed that some exposed fish responded to a greater degree than others, while some did not respond at all.

Chromosomal protein analysis - Total staining of red blood cell chromatin proteins revealed that a wide suite of proteins, ranging in size from 14 to greater than 100 kDa, showed striking changes in temporal patterns of expression in exposed fish. These data are summarized in Table 9. After 1 week of exposure, there was a decrease in the concentration of most chromatin proteins of exposed fish compared to controls. By the second week, and continuing through week four, there seemed to be a general increase in expression of chromatin proteins in exposed fish. Proteins in exposed fish were once again depressed at week 8. The data from weeks 16 and 40 indicate a general induction of proteins, except for those > 100 kDa, which showed a decrease in exposed fish. In addition, the proteins tentatively identified as histones H1 and H5 showed a shift from three bands in control fish to two

in the exposed resulting from loss of putative H5 histone. This may indicate a change in the state of red cell differentiation in exposed fish. In vertebrates with nucleated red cells, there is an increase in the portion of H5 histone relative to the related H1 histone as erythrocytes age and the nuclei become pycnotic (Palyga 1990, Greenaway and Murray 1971). Therefore, in exposed fish a loss of older mature erythrocytes may occur concurrent with increased recruitment from precursor cells in exposed fish. At week 40, the protein bands tentatively identified as the histone H2a-H2b-H3-H4 nucleosome complex showed a change from the two intense bands observed in control fish to three in the exposed. The histones were identified by their migration in SDS gels relative to chicken erythrocyte standards and by the fact that these protein bands comprise greater than 50% of chromosomal protein in fish erythrocyte nuclei. The nucleosome histone densitometric profiles were invariant for both control and exposed fish except for week 40. The alterations seen at week 40 may be the result of histone modification and changes in chromatin packaging or structure possibly as a result of a change in the composition of red cell maturational stages or toxicological effects on erythrocytes.

Interestingly, the gill stress proteins and blood chromatin proteins exhibit temporal responses similar to each other and to the trends of DNA damage. At present, however,

the precise relationship between stress proteins, chromatin proteins and strand breakage is not known but the chromatin proteins of molecular weights 43, 50, 60, 70 and 78 kDa (Table 9) were highly expressed in exposed fish relative to controls at week 40. A number of stress proteins are homologous in molecular weight to those (70 kDa) induced in this study. Specific antibody probes to stress/heat shock proteins may be used to identify the class of proteins induced in fish exposed to anthropogenic agents. To our knowledge, there is no other study which has used fish blood chromatin proteins as a biomarker of exposure.

**Comparisons Between Biomarkers**

A significant aspect of this study is that patterns of biochemical and molecular responses are also seen at the organ or tissue level. For example, both EROD activity and liver somatic indices (LSI) show a trend of increasing magnitude of the response with time (Fig. 3). Both factors seem to be reliable indicators of liver damage, since focal or single cell necrosis also occurred in fish with elevated EROD and LSI values. Furthermore, skeletal muscle concentrations of PCBs also increase with time. Although liver concentrations of PCBs were not determined, it may be assumed that they too increased over time, and this may be a partial explanation for increasing values for EROD and LSI over time.

Also, ATPase activity seems to be a good correlate of tissue damage. For example, depression of ATPase activity of the gill was apparent after only one week, and the relative amount of inhibition remained stable over time (Fig. 2). The same was true of tissue damage to the gill. In the kidney, on the other hand, both ATPase depression (Fig. 6) and tubular damage were apparent only after long-term exposures.

Molecular responses at the DNA level also paralleled the cytological responses. In comparing the results from flow cytometry and the alkaline unwinding assays, it can be seen that the amount of cell-to-cell variation in DNA content is lowest at 8 weeks, and that the number of DNA strand breaks in exposed fish is also lowest at 8 weeks (Table 7). This suggests some relationship between these two parameters, the common factor probably being DNA damage and/or repair, but the molecular mechanism behind this relationship remains to be elucidated.

It is also interesting to note that different biochemical markers from the same organ show similar patterns. For instance, both brain ATPase and AchE activities are initially induced in exposed fish relative to controls, but are latter inhibited (Fig. 1). This is not surprising, since both enzymes are membrane-bound proteins found in high concentrations in neuronal tissue (Alberts et al. 1983).

Also, organs which share related functions show similar trends of response. In this respect, both the kidney and the

spleen are involved in hematopoiesis in fish (Gromman 1982). Both organs also show delayed responses to toxicants, indicating a general disruption of hematopoietic function after chronic exposure. ZPP levels also increased dramatically after chronic exposure (Fig. 6), and this may be a correlate of hematopoietic function. Two other correlates of hematopoiesis, ALAD activity (without chelators) and hematocrit, show parallel temporal patterns of response. Both biomarkers in exposed fish are equal to or slightly greater then those of control fish after one week, but then fall below controls thereafter (Table 8, Fig. 6). This is understandable, since ALAD is the rate-limiting step in the synthesis of heme (Labbe and Rettmer 1989), and is an essential component of blood formation.

The results from some organs, on the other hand, show different patterns of response than others. DNA adducts in the gill and blood appear earlier and at larger concentrations than in the liver (Table 7). The rapid accumulation of DNA adducts in blood compared to gill may reflect the fact that mature red blood cells lose their ability to synthesize DNA (Mahajan and Deer 1980), so they may have reduced DNA repair capabilities as well.

Generally, the fact that biomarker responses follow similar trends implies that information from one marker or organ can infer information about other markers or organs. One example is that depression of ATPase activity seems to be

closely correlated with tissue damage. As another example, DNA-BaP adducts in the blood seem to be an early indication that these adducts will show up in the liver at a later time point.

Biomarkers in the gill, intestine, and blood seem to respond quickly to contaminants. In general responses remaining relatively stable over time with the exceptions of stress and chromatin proteins. Biomarkers in organs which are internal to the site of exposure (brain, kidney, liver, spleen) show increasing responses over time. Several factors could play a role in these phenomena, for example: 1) The gill and intestine are directly exposed to the toxins. 2) There may be tissue-specific differences in the amount of resistance to or recovery from the action of toxicants. 3) The response of internal organ alterations over time may be due to accumulated injury or to the action of compensatory systems which can cope with short- but not long-term insult. 4) Xenobiotics may equilibrate within the various tissues at different rates. Heavy metals reached equilibration relatively quickly, but PCB concentrations increased over time. This may be an important consideration in fatty tissues, such as the liver and brain. 5) Physiological acclimation to the toxic environment may result in shifting patterns of responses.

**Comparisons to EFPC Studies**

In general, results from the longer laboratory exposures more closely resemble those found in EFPC fish. This is particularly true for DNA strand breakage and, liver mixed-function oxidase. Although these responses in the laboratory are qualitatively similar to those in redbreast sunfish in EFPC, the magnitude of the response is much greater in EFPC fish than in the laboratory (Table 10). This could be due to the fact that the EFPC fish are subjected to lifetime exposures, or differences between bluegill and redbreast sunfish. In addition, it has been found that certain biomarkers (e.g., DNA strand breaks) show a much more marked response to toxicants when the animals are stressed with temperature or organic solvents (Shugart 1988). In the laboratory, the fish are kept at a constant temperature, fed ad libitum, and supplied with water that is fully saturated with air. Thus for fish in the lab the number of stressors other than xenobiotic stress are kept to a minimum, while this is not true for EFPC fish. Natural selection, population and community interactions, abiotic mediating factors in the field, or any combination thereof could also affect these responses.

In addition, the variability of the response between individuals may reflect genetic differences in the ability to resist or adapt to toxicological stress. In many cases, the variability in unexposed animals is somewhat less than in exposed animals (eg., see Figs. 3 and 4). In fact, increased

variability in responses has been proposed as a biomarker in itself (M.S. Adams, Environmental Sciences Division, ORNL; personal communication). Obviously, inherent differences in the resistance to stressors will not become apparent until the fish are stressed. Individuals which are more adept at compensating for any physiological perturbation, however slight, would have a selective advantage over others in the population.

**Comparisons to Other Studies**

This study is also significant in that it is an important link between single-contaminant laboratory studies and field studies, which often examine a wide suite of organic and inorganic chemicals. Note that biomarkers seen with exposure to single toxicants are also seen when the fish are exposed to EFPC sediment. For example, BaP adducts are a specific marker for BaP exposure (Shugart 1987). In this study, the fish were exposed to BaP as well as numerous other PAHs, organic, and inorganic compounds, but still displayed responses similar to single-exposure experiments.

Another significant aspect of this study is that the results for some bluegill biomarkers show the same trends as results from other fishes or even other vertebrates, for example, rodents (McBee and Bickham 1988, Shugart 1987), turtles (Bickham et al. 1988) or herons (see above). This is

an important component of linking biomarker data with community and ecosystem level responses. Also, the ecological relevance of single-species tests is strengthened by the fact that results from one species are applicable to other species. In particular, this implies that every species in an ecosystem does not need to be sampled in order to demonstrate an effect of anthropogenic contamination on that ecosystem.

As a final note, we would like to point out that there are water-associated factors (e.g. $Ca^{+2}$ concentration, water hardness, pH, and dissolved organic carbon) which may potentiate the availability and toxicity of xenobiotics in the sediment. These factors may modulated xenobiotic interactions between the sediment and water (e.g. partitioning, adsorption/desorption, etc.). In this study, as in field studies, these interactions were allowed to take place during exposure of the fish.

## CONCLUSIONS

1) Laboratory results using contaminated sediment parallel those found in fish collected from the site of the sediment's origin. This illustrates that laboratory and field studies supplement each other. The responses of fish exposed to the sediment in the lab are similar to but lesser in magnitude than EFPC fish, perhaps due to species-specific differences, lifetime toxicant exposures, natural selection, population and community interactions, or abiotic mediating factors in the field.

2) The duration of a laboratory exposure requires consideration, since different biomarkers show different trends of response over time. Choosing a biomarker appropriate for the time course of the study is an important consideration. Certain biomarkers are affected by xenobiotics but remain stable over time, so they should provide comparable field and short-term laboratory data. Some biomarker responses fluctuate over time, while others show steady time-dependant increases. For these markers only long-term laboratory data may be comparable to the field.

3) Variation in biomarker response can be quite large. This may have a genetic basis and could have evolutionary and ecological implications.

4) Certain biomarkers show very similar trends over time, while others are quite different. This has to be taken into

account when deciding which organ or organ system to use as biomarkers. Overall, the blood and the organs at the site of exposure seem to respond quicker and with greater magnitude than internal organs. Internal organs seem to be more indicative of chronic exposures. Generally, the blood seems to be a good indicator of stress, and has the advantages that it is easy to collect and data can be recorded from live specimens. Thus, data can be collected from one fish over several points in time, which may reduce individual variability in time-course experiments. Also, for field studies, animals do not need to be permanently removed from the ecosystem. Finally, non-lethal biomonitoring may seem more acceptable to certain aspects of society.

**ACKNOWLEDGEMENTS**

The authors would like to graciously thank the following people for their assistance for laboratory analyses: R. G. Epler, M. K. McCracken, C. Gonzalez, D. Taylor, C. E. Guzman, M. Mascerinek, K. J. Wilkinson and B.L. Lowe. Portions of this study were presented at the 11th Annual Conference of the Estuarine Research Federation, San Francisco, November 10-14, 1991. This project was sponsored in part by the Oak Ridge Y-12 Plant, Department of Environmental Management, Health, Safety, Environment and Accountability Division and by an appointment to the U.S. Department of Energy Laboratory Cooperative Postgraduate Research Training Program administered by the Oak Ridge Associated Universities. The Oak Ridge National Laboratory is operated by Martin Marietta Energy Systems under Contract DE-AC05-84OR21400 with the U.S. Department of Energy. This paper is contribution #4 of the Center for Biosystematics and Biodiversity, Texas A&M University, a facility funded in part by the National Science Foundation (DIR-8907006), and is Environmental Sciences Division publication no. 3923.

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## APPENDIX

Table 1 - Suite of Biomarkers examined in bluegill sunfish exposed to EFPC sediment.

| Biomarker* | Tissue** | Contaminant/Reference† |
|---|---|---|
| $Na^+/K^+$ ATPase | B,G,I,K | metals & PCBs/Lakshmi et al., 1991; La Roca and Carlson, 1979 |
| AchE | B | metals & PAHs/Shaw & Panigrahi 1990; Danga & Masurekar, 1989 |
| EROD/cytochrome $P_{450}$/microsomal NADH/NADPH | L | PAHs & PCBs/Adams et al., 1990 |
| Liver somatic index | L | PAHs & PCBs/Adams et al, 1990; Fletcher et al., 1982 |
| Spleen somatic index | S | metals & hydrocarbons/Lowe-Jinde & Niimi, 1986; Adams et al., 1990 |
| PAH metabolites | bile | Krahn et al., 1984 |

Table 1 (cont')

| Biomarker* | Tissue** | Contaminant/Reference† |
|---|---|---|
| DNA: strand breaks | L | Shugart, 1988 & 1990 |
| DNA: B[a]P adducts | L,G,rbc | Shugart, 1987 |
| Hb: B[a]P adducts | rbc | Shugart, 1985 & 1987 |
| DNA content | L | metals & PAHs/Bickham, 1990 |
| Histopathology | G,K,L | metals & PAHs/Nath & Kumor, 1988; Kinubagaran & Joy, 1988 |
| Stress proteins | G | metals/Bradley, 1992 |
| Chromatin proteins | rbc | PAHs/Theodorakis & D'Surney, unpublished data |

*Abbreviations: AchE, Acetylcholinesterase activity; ALAD, δ-amino levulenic acid dehydratase activity; EROD, Ethoxyresorufin-o-deethylase activity; Hb, Hemoglobin; Hct, Hemotocrit; NADH, NADH-cytochrome $b_5$ reductase activity; NADPH, NADPH-cytochrome c reductase activity; ZPP, Zinc protoporphyrin content of the blood.

Table 1 (cont')

**Tissue in which biomarker examined (B, brain; G, gill; I, intestine; K, kidney; L, liver; rbc, red blood cell)

†Environmental contaminants known to elicit a change in biomarker response.

Table 2 - Water quality parameters for water equilibrated with EFPC sediment.

| | | Sediment | |
|---|---|---|---|
| Parameter | Week | Control | EFPC |
| Temperature | 1 | 21 | 21 |
| (°C) | 2 | 20 | 21 |
| | 4 | 21 | 21 |
| | 8 | 21 | 21 |
| pH | 1 | 8.1 | 8.2 |
| | 2 | 8.5 | 8.3 |
| | 4 | 8.2 | 8.0 |
| | 8 | 8.0 | 8.1 |
| Total inorganic | 1 | 7.59 | 8.36 |
| carbon (mg/L) | 2 | 7.89 | 7.98 |
| | 4 | 6.99 | 7.89 |
| | 8 | 7.45 | 8.01 |
| Calcium (mg/L) | 1 | 54.0 | 34.0 |
| | 2 | 55.0 | 44.0 |
| | 4 | 60.0 | 47.0 |
| | 8 | 52.0 | 39.0 |

Table 2 (cont')

| Parameter | Week | Control | EFPC |
|---|---|---|---|
| Total organic | 1 | 8.65 | 8.40 |
| carbon (mg/L) | 2 | 6.82 | 5.49 |
| | 4 | 6.13 | 5.01 |
| | 8 | 6.84 | 5.23 |

Table 3 - Representative PCBs, PAHs and heavy metals found in EFPC sediment.

| Compound | Sediment | | Worms |
|---|---|---|---|
| | Control | EFPC | |
| **PCBs** (µg per g wet weight)** | | | |
| 22′ DCB | --† | 205.24 | 7.98 |
| 24 DCB | -- | 31.98 | 7.97 |
| 44 DCB | -- | 9.48 | 2.62 |
| 246 TCB | -- | 22.06 | 0.36 |
| 22′4 TCB | -- | 78.12 | 0.67 |
| 22′5 TCB | -- | 100.47 | 1.99 |
| 22′55′ TtCB | -- | 13.28 | 20.2 |
| 22′455′ PCB | -- | 5.56 | 2.67 |
| 22′466′ PCB | -- | 5.49 | 0.86 |
| 22′33′44′ HCB | -- | 4.08 | 2.15 |
| 22′44′55′ HCB | -- | 2.19 | 1.38 |
| 22′44′66′ HCB | -- | 11.04 | 1.21 |
| Total Aroclors‡ | 0.49 | 7.78 | -- |

Table 3 (cont')

| Compound | Sediment | | Worms |
|---|---|---|---|
| | Control | EFPC | |
| **PAHs (μg per g wet weight)** | | | |
| Fluorene | ND* | 9.10 | 1.48 |
| Phenanthrene | ND | 22.98 | 4.40 |
| Anthracene | ND | 22.90 | 4.43 |
| Benzanthracene | ND | 2.97 | 1.61 |
| Chrysene | ND | 98.01 | 11.89 |
| Pyrene | ND | 120.80 | 10.93 |
| Benzo [k] | ND | 2.17 | 0.98 |
| fluoranthrene | ND | 66.56 | 7.72 |
| Benzo [a] pyrene | ND | 14.33 | 2.48 |
| Dibenz [a,h] anthracene | | | |
| **Heavy metals (ng per g wet weight)** | | | |
| Al | 370 | 530 | 208 |
| As | 6.1 | 85 | 1.26 |
| Cu | 3.6 | 140 | 0.71 |
| Ni | 8.6 | 64 | 8.86 |
| Pb | 6.1 | 75 | 1.26 |
| Zn | 83.0 | 890 | 6.42 |

*Not detected.

Table 3 (cont')

**DCB - di-chlorobiphenyl; TCB - trichlorobiphenyl; TtCB - tetrachlorobiphenyl; PCB - pentachloro-biphenyl; HCB - hexachlorobiphenyl.

†Indicates that this particular assay was not performed.

‡Indicates that PCBs were identified as Aroclors rather than individual congeners.

Table 4 - PCB, PAH and heavy metal concentrations (μg/L) of water equilibrated with EFPC sediment.

| Compound | Week 1 | Week 2 | Week 4 | Week 8 |
|---|---|---|---|---|
| **PCB Congeners*** | | | | |
| 22′ DCB | 220.6 | 62.0 | 52.2 | 57.8 |
| 24 DCB | 55.0 | 13.0 | 18.2 | 15.4 |
| 44 DCB | 42.5 | 8.2 | 7.6 | 7.5 |
| 246 TCB | 5.6 | 1.4 | 0.9 | 0.8 |
| 22′4 TCB | 2.6 | 0.8 | 1.0 | 0.7 |
| 22′5 TCB | 3.0 | 3.0 | 0.7 | 1.3 |
| 22′55′ TtCB | 20.0 | 2.8 | 2.8 | 2.3 |
| 22′455′ PCB | 7.4 | 0.9 | 0.7 | 0.8 |
| 22′466′ PCB | 10.7 | 0.7 | 1.2 | 1.0 |
| 22′33′44′ HCB | 17.2 | 0.4 | 0.3 | 0.3 |
| 22′44′55′ HCB | 11.5 | 4.4 | 0.3 | 0.4 |
| 22′44′66′ HCB | 5.2 | 0.6 | 0.7 | 0.6 |

Table 4 (cont')

| Compound | Week 1 | Week 2 | Week 4 | Week 8 |
|---|---|---|---|---|
| **PAH Congeners** | | | | |
| Fluorene | 0.5 | 0.5 | 0.4 | 0.3 |
| Phenanthrene | 2.1 | 2.6 | 2.3 | 2.2 |
| Anthracene | 1.2 | 1.1 | 1.1 | 1.2 |
| Benzanthracene | 0.1 | 0.1 | 0.04 | 0.04 |
| Chrysene | 6.8 | 5.4 | 3.3 | 2.8 |
| Pyrene | 4.1 | 3.8 | 3.0 | 5.5 |
| Benzo [k] fluorathrene | 0.04 | 0.04 | 0.04 | 0.2 |
| Benzo [a] pyrene | 0.6 | 0.6 | 0.2 | 0.5 |
| Dibenz [a,h] anthracene | 0.1 | 0.1 | 0.1 | 0.2 |
| **Heavy Metal** | | | | |
| Al | 60 | 50 | 57 | 110 |
| As | < 50** | < 50** | 50 | < 50** |
| Cd | < 7** | 7** | 7 | < 7** |
| Cu | 16 | 7 | 8 | < 5** |
| Ni | 12 | 4 | 7 | 6 |
| Pb | 50 | 30 | 32 | 34 |
| Zn | 54 | 24 | 22 | 18 |

Table 4 (cont')

*DCB - di-chlorobiphenyl; TCB - trichlorobiphenyl; TtCB - tetrachlorobiphenyl; PCB - pentachloro-biphenyl; HCB - hexachlorobiphenyl.

**Detection limit.

Table 5. Median (range) PCB concentrations in bluegill sunfish exposed to water equilibrated with EFPC sediment.

| Congener* | PCB Concentration (μg PCB per g wet weight) | | | BCF** |
|---|---|---|---|---|
| | Week 1 | Week 2 | Week 4 | |
| 22′ DCB | 2.93 (1.89-3.11) | 7.09 (4.46-9.71) | 37.90 (37.06-38.81) | 726 |
| 24 DCB | 0.36 (0.24-0.48) | 1.64 (1.36-1.92) | 7.03 (6.19-8.78) | 386 |
| 44 DCB | 1.69 (0.89-1.89) | 3.59 (3.11-5.06) | 22.77 (16.13-30.65) | 2,996 |
| 22′4 TCB | 1.42 (0.91-1.67) | 0.63 (0.29-0.86) | 3.40 (1.92-3.96) | 3,400 |
| 22′5 TCB | 2.14 (0.96-2.98) | 0.88 (0.62-1.14) | 1.42 (1.01-1.52) | 2,029 |
| 22′55′ TtCB | 0.28 (0.09-0.56) | 1.15 (1.17-1.23) | 7.99 (3.42-9.73) | 2,854 |
| 22′455′ PCB | 0.99 (0.30-1.09) | 1.17 (1.07-1.19) | 2.46 (2.97-2.95) | 3,514 |
| 22′466′ PCB | 1.02 (0.60-1.34) | 1.13 (1.09-1.16) | 3.73 (3.61-11.01) | 3,108 |
| 22′33′44′ HCB | 0.32 (0.05-0.45) | 2.15 (2.10-2.19) | 6.13 (6.09-6.39) | 20,433 |
| 22′44′55′ HCB | 0.36 (0.09-0.43) | 1.30 (1.24-1.36) | 3.38 (3.24-3.47) | 11,267 |
| 22′44′66′ HCB | 0.29 (0.08-0.46) | 0.63 (0.52-0.74) | 1.43 (1.31-3.37) | 2,024 |

Table 5 (cont')

| Congener* | PCB Concentration (μg PCB per g wet weight) Week 1 | Week 2 | Week 4 | BCF** |
|---|---|---|---|---|
| Σ PCB | 12.00 | 20.12 | 97.64 | 10,000 |

*DCB - di-chlorobiphenyl; TCB - trichlorobiphenyl; TtCB - tetrachlorobiphenyl; PCB - pentachloro-biphenyl; HCB - hexachlorobiphenyl

**Bioconcentration factor: concentration in the fish (μg/kg) ÷ concentration in the water (μg/l). The bioconcentration factor was calculatied for the highest concentration (i.e. week 4) only.

Table 6. Median (range) concentration of heavy metals in bluegill sunfish tissue exposed to water equilibrated with EFPC sediment.

| | Metal Concentration (ng/g wet weight) | | | | |
|---|---|---|---|---|---|
| | Week 1 | Week 2 | Week 4 | Week 8 | Week 16 |
| Al | 43 (30-56) | 46 (32-110) | 42 (30-53) | 49 (126-52) | 41 (85-97) |
| As | 76 (49-80) | 60 (57-91) | 50 (41-59) | 95 (49-140) | 80 (23-56) |
| Cd | 13 ( 7-17) | 20 ( 8-93) | 18 (10-26) | 17 ( 5-28) | 9 ( 3-14) |
| Cu | 37 (24-157) | 26 (19-106) | 20 ( 7-32) | 19 (14-23) | 16 ( 6-26) |
| Ni | 49 (10-92) | 32 (13-45) | 34 (27-40) | 55 (39-70) | 29 (24-33) |
| Pb | 65 (38-88) | 86 (35-163) | 71 (33-109) | 88 (25-150) | 95 (50-140) |
| Zn | 99 (43-54) | 109 (89-128) | 79 (42-115) | 89 ( 5-73) | 96 (49-142) |

Table 7 - Biomarkers indicative of exposure to genotoxic agents in tissues of bluegill sunfish exposed to EFPC sediment.

| Tissue | Week | Sediment* | |
|---|---|---|---|
| | | Control | EFPC |
| **PAH Bile Metabolites (μg/mL bile)** | | | |
| Bile | 1 | 0.20 (0.19-0.21) | 67.1 (62.1-86.8)§ |
| | 4 | 0.19 (0.17-0.19) | 61.9 (47.1-63.2)§ |
| | 8 | 0.20 (0.19-0.23) | 63.1 (57.0-69.3)§ |
| | 16 | 0.17 (0.16-0.19) | 61.9 (55.3-64.4)§ |
| **DNA Strand Breaks (F values)** | | | |
| Liver | 1 | 0.71 (0.59-0.76) | 0.46 (0.33-0.50)§ |
| | 2 | 0.58 (0.49-0.59) | 0.56 (0.55-0.58) |
| | 8 | 0.65 (0.47-0.69) | 0.84 (0.74-0.94)§ |
| | 16 | 0.68 (0.55-0.70) | 0.35 (0.28-0.42)§ |

Table 7 (cont')

| Tissue | Week | Sediment* Control | Sediment* EFPC |
|---|---|---|---|
| **DNA Content**** | | | |
| Liver | 1 | 65.0 (60.8-69.1) | 60.9 (58.8-64.2) |
| | 4 | 66.9 (66.4-69.0) | 65.8 (65.7-65.9) |
| | 8 | 66.7 (60.8-69.1)† | 61.1 (60.7-61.3)§ |
| | 40 | 66.8 (61.0-68.3)† | 62.5 (61.9-64.6)§ |
| **DNA Cell-to-Cell Variability*** | | | |
| Liver | 1 | 4.5 (4.3-4.6) | 5.6 (4.6-6.1)‡ |
| | 2 | 4.8 (4.8-5.7) | 5.8 (5.4-6.3)‡ |
| | 8 | 4.7 (3.5-5.7) | 3.9 (3.8-4.4) |
| | 40 | 4.7 (4.5-4.8) | 5.3 (4.8-5.7)‡ |
| **DNA-BaP Adducts (ng adduct/g DNA)** | | | |
| Liver | 1 | ND* | ND |
| | 2 | ND | ND |
| | 40 | ND | 2.60 (1.42-2.95) |

Table 7 (cont')

| Tissue | Week | Sediment* | |
|---|---|---|---|
| | | Control | EFPC |
| **Hemoglobin-BaP Adducts (ng adduct/g hemoglobin)** | | | |
| Blood | 1 | ND | 0.02 (0.01-0.03) |
| | 2 | ND | |
| | 40 | ND | 0.11 (0.10-0.12) |
| | | | 0.12 (0.05-0.18) |

*Values indicate medians with ranges in parentheses.

**Determine by flow cytometry as mean amount of DNA/cells in $G_1$; units = relative fluorescence units.

†Samples for which n (number of samples) = 6, in all other cases n = 3.

‡$P \leq 0.10$, Wilcoxon Rank Sum Test

§$P \leq 0.05$, Wilcoxon Rank Sum Test

Table 7 (cont')

*Determined by flow cytometry as coefficient of variation of the mean amount of DNA/cell; units = relative fluorescence units.

*Not detected.

Table 8. δ-Amino levulinic acid dyhydratase activity for bluegill sunfish exposed to EFPC sediment.

| Activity (nM / ml red blood cell / hr) | | | | |
|---|---|---|---|---|
| | | Sediment* | | Alpha |
| Treatment | Week | Control | EFPC | Value** |
| With | 2 | 888 (789-974) | 985 (931-2273) | 0.10 |
| chelators† | 4 | 753 (699-753) | 1238 (769-1486) | 0.05 |
| | 8 | 750 (626-874)‡ | 990 (877-1047) | 0.10 |
| | 16 | 801§ | 943§ | --- |
| Without | 1 | 867 (863-914) | 917 (823-1081) | 0.35 |
| chelators | 2 | 852 (652-922) | 372 (265-578) | 0.05 |
| | 8 | 899 (737-1046) | 712 (688-753) | 0.10 |

*Values indicate medians with the ranges in parentheses. n = 3 for all samples unless otherwise indicated.

Table 8 (cont')

**Probability levels for the Wilcoxon rank sum test.

†Metal chelators used in the assay were EDTA and citrate. Chelation of metals could affect inhibition of ALAD by lead.

‡Indicates a sample with sample size of 2.

§Indicates a sample with sample size of 1.

Table 9. Comparison of the relative intensity of chromosomal protein bands* from bluegill sunfish exposed to EFPC sediment, examined in a polyacrylamide electrophoretic gel.

| | Week | | | | | |
|---|---|---|---|---|---|---|
| Protein | 1 | 2 | 4 | 8 | 16 | 40 |
| H2-H3** | ND† | ND | ND | ND | ND | 2→3‡ |
| 20 kDa§ | C>E | ND | ND | C>E | ND | E>>C |
| H1-H5 | 3→2* | ND | ND | 3→2 | 3→2 | 3→2 |
| 36-42 kDa‡ | C>E | ND | C>E | C>E | ND | C>E |
| 43 kDa | C>E | ND | ND | E<C | E>C | E>>C |
| 50 kDa | C>E | E>>C | ND | C>E | C>E | E>>C |
| 60 kDa | C>E | ND | ND | C>E | ND | E>>C |
| 70 kDa | C>E | E>>C | E>>C | C>E | C>E | E>>C |
| 78 kDa | C>E | E>C | E>>C | C>E | E>>C | E>>C |
| 48-97 kDa‡ | C>E | E>C | C>E | C>E | E>C | E>C |
| >100‡ | C>E | E>>C | C>E | C>E | C>E | C>E |

*"E>C" signifies that this band is more intense in exposed fish, "E>>C" signifies that this protein band is much more intense in exposed fish, "C>E" signifies that this protein band is more intense in control fish.

**Indicates a histone complex. H1 is not a core histone as are histones H2 and H3. Histone H5 is a histone that is

Table 9 (cont')

normally found only in the erythrocytes of non-mammmalian vertebrate animals. These histones have similar molecular weights, so they could not be resolved using the type of electrophoresis used in this study.

†No difference between control and exposed fish.

‡Indicates a condition where there are 2 protein bands in this complex for control fish and 3 protein bands for exposed fish.

§Identifies an unknown protein by approximate molecular weight (kiloDaltons, kDa).

*Indicates a condition where there are 3 protein bands in this complex in control fish and 2 protein
bands in exposed fish.

⁑A suite of protein bands not previously identified, but showing similar patterns.

Table 10. Summary of values for biomarkers from redbreast sunfish living in East Fork Poplar Creek.

| Marker (units) | Field data* | | Laboratory data** | |
|---|---|---|---|---|
| | Reference Stream | EFPC | Control Sediment | EFPC Sediment |
| Microsomal cytochrome $P_{450}$ content (pmole/mg protein) | 400 | 800 | 80 | 120 |
| Microsomal cytochrome $b_5$ content (pmole/mg protein) | 40 | 100 | 275 | 325 |

Table 10 (cont')

| Marker (units) | Field data* | | Laboratory data** | |
|---|---|---|---|---|
| | Reference Stream | EFPC | Control Sediment | EFPC Sediment |
| Microsomal NADH-cytochrome $b_5$ reductase activity (nmole/mg protein/min) | 230 | 380 | 60 | 90 |
| Microsomal NADPH-cytochrome c reductase activity (nmole/mg protein/min) | 90 | 170 | 110 | 120 |

Table 10 (cont')

| Marker (units) | Field data* | | Laboratory data** | |
|---|---|---|---|---|
| | Reference Stream | EFPC | Control Sediment | EFPC Sediment |
| EROD activity (pmol/mg protein/min) | 2 | 120 | 5 | 50 |
| Liver Somatic Index (% body mass) | 1.0 | 1.6 | 1.0 | 2.0 |
| Strand breaks (N value) | --- | 4.0 | --- | 1.7 |

*Sources:

Loar J.M., S.M. Adams, H.L. Boston, B.D. Jimenez, J.F. McCarthy, J.G. Smith, G.R. Southworth, and A.J. Stewart. 1988. First annual report on the Y-12 plant biological monitoring and abatement

Table 10 (cont')

program. Oak Ridge: ORNL/TM, Oak Ridge National Laboratory.

Jimenez B.D., L.S. Burtis, G.H. Ezell, B.Z. Egan, N.E. Lee, J.J. Beauchamp and J.F. McCarthy. 1988. The mixed function oxidase system of bluegill sunfish, Lepomis macrochirus: Correlation of activities in experimental and wild fish. Environ. Toxicol. Chem. 7: 623-634.



Shugart , L.R. 1988. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Toxicol. 13: 43-52.

**Source: This study at longest exposure period.

**Figure Legends**

1. ATPase (A) and Acetylcholinesterase (B) activities from the brains of bluegill sunfish exposed to contaminated water. Water was contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars represent the range. * $P < 0.10$; ** $P < 0.05$.

Figure 2 - $Na^+/K^+$ ATPase activities from the gills (A) and intestines (B) of bluegill sunfish exposed to contaminated water. Water was contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars represent the range. * $P < 0.10$; ** $P < 0.05$.

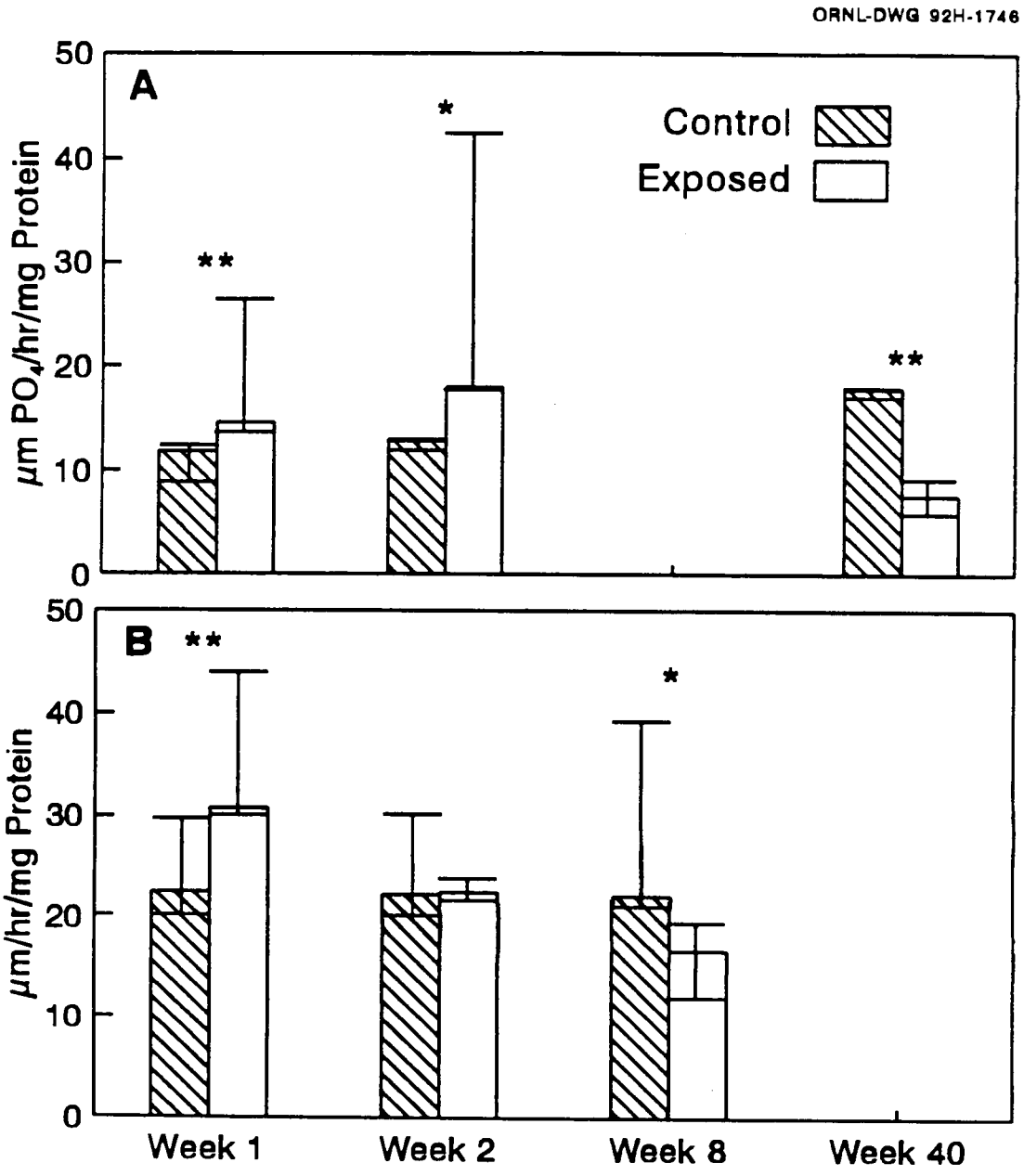
Figure 3 - Ethoxyresorufin-o-deethylase (EROD) activity (A) and liver somatic index (B) from the livers of bluegill sunfish exposed to contaminated water. Water was contaminated with PAHs, PCBs and heavy metals over several weeks. Error bars represent the range. * $P < 0.10$; ** $P < 0.05$.

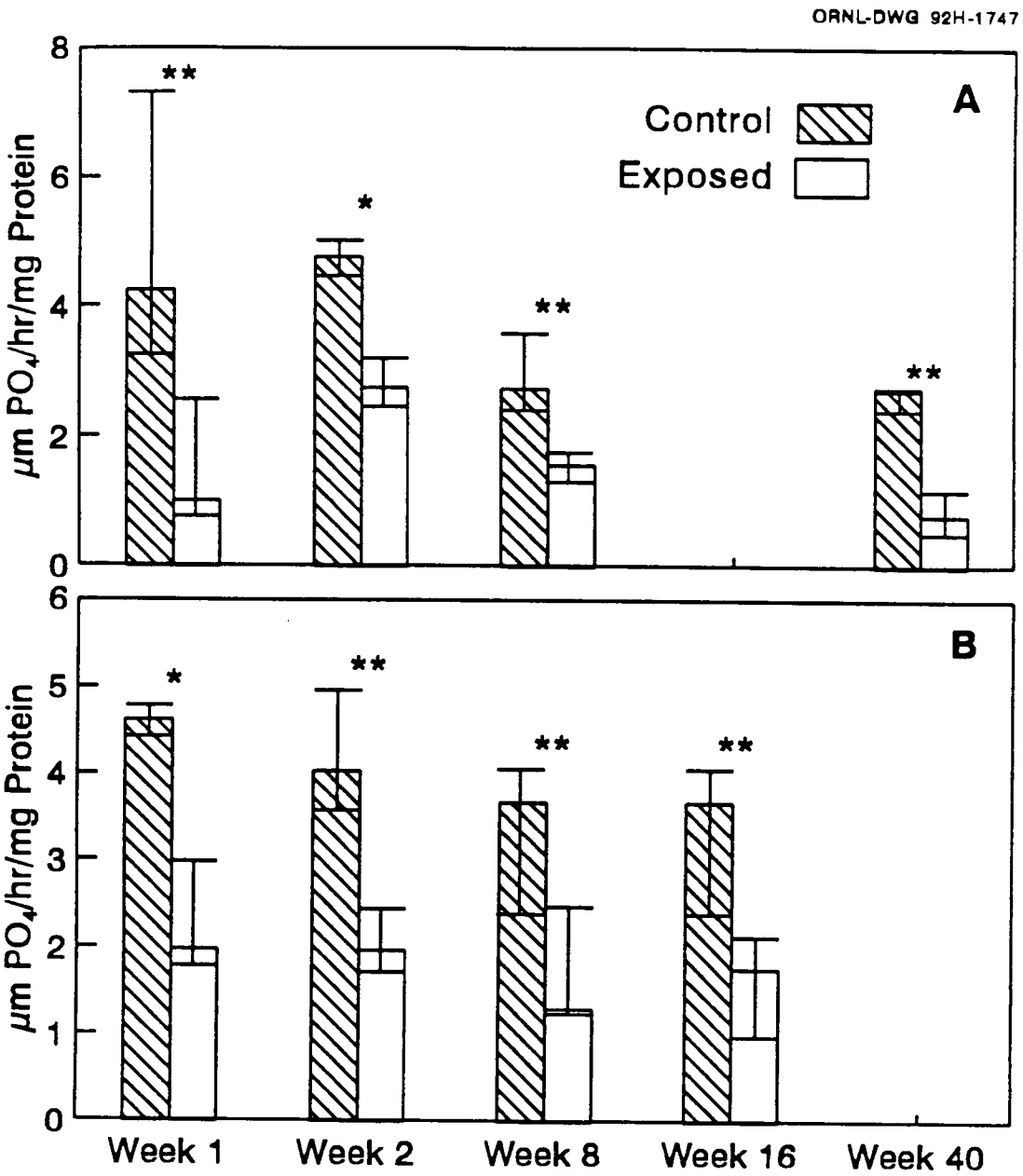
Figure 4 - Microsomal cytochromes $P_{450}$ (A) and $b_5$ (B) concentrations, NADH-cytochrome $b_5$ reductase activity (C) and NADPH cytochrome c reductase activity (D) from the livers of bluegill sunfish. Fish were exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both

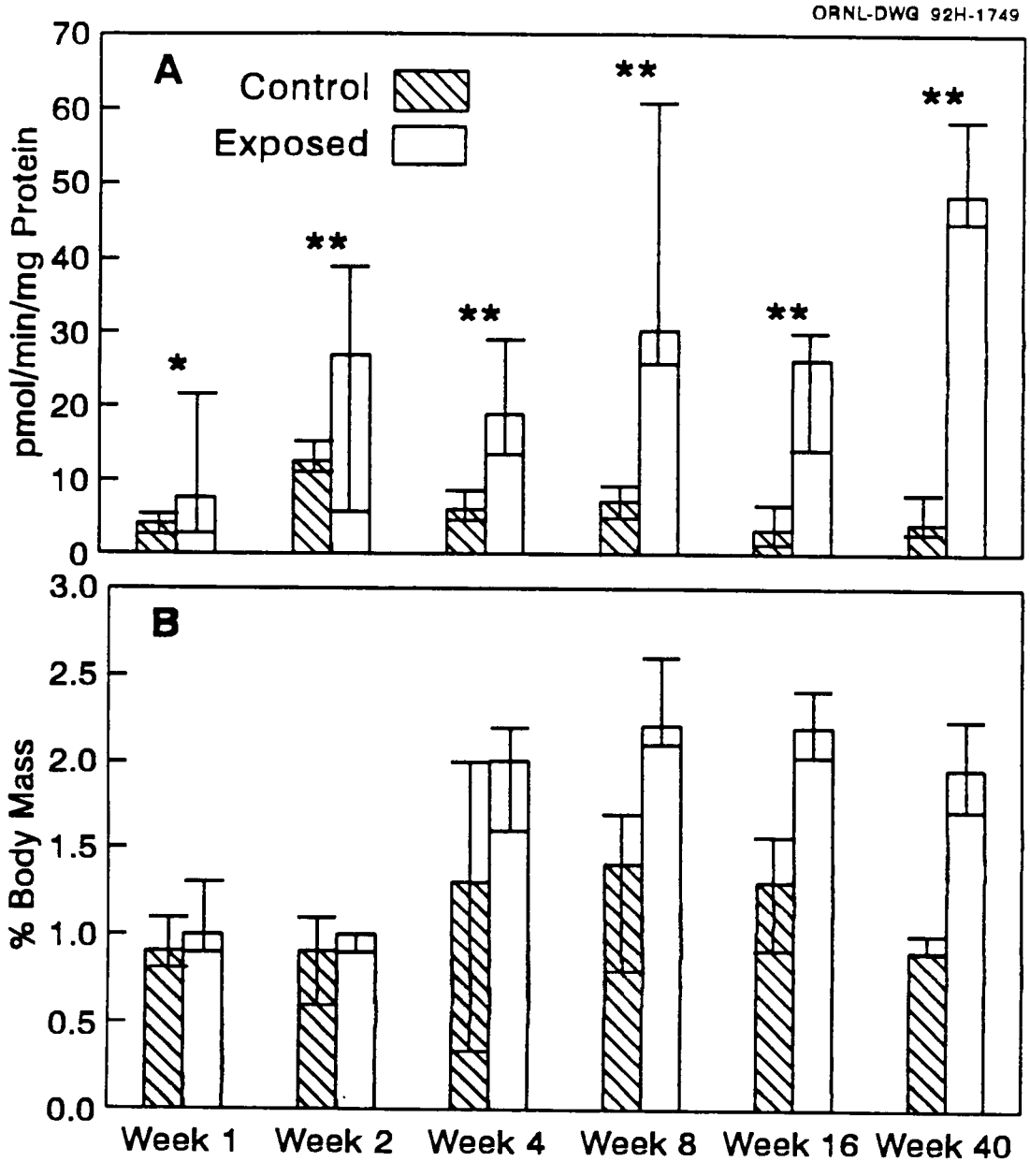
control and exposed fish are indicated. Error bars represent the range. * $P < 0.10$; ** $P < 0.05$.

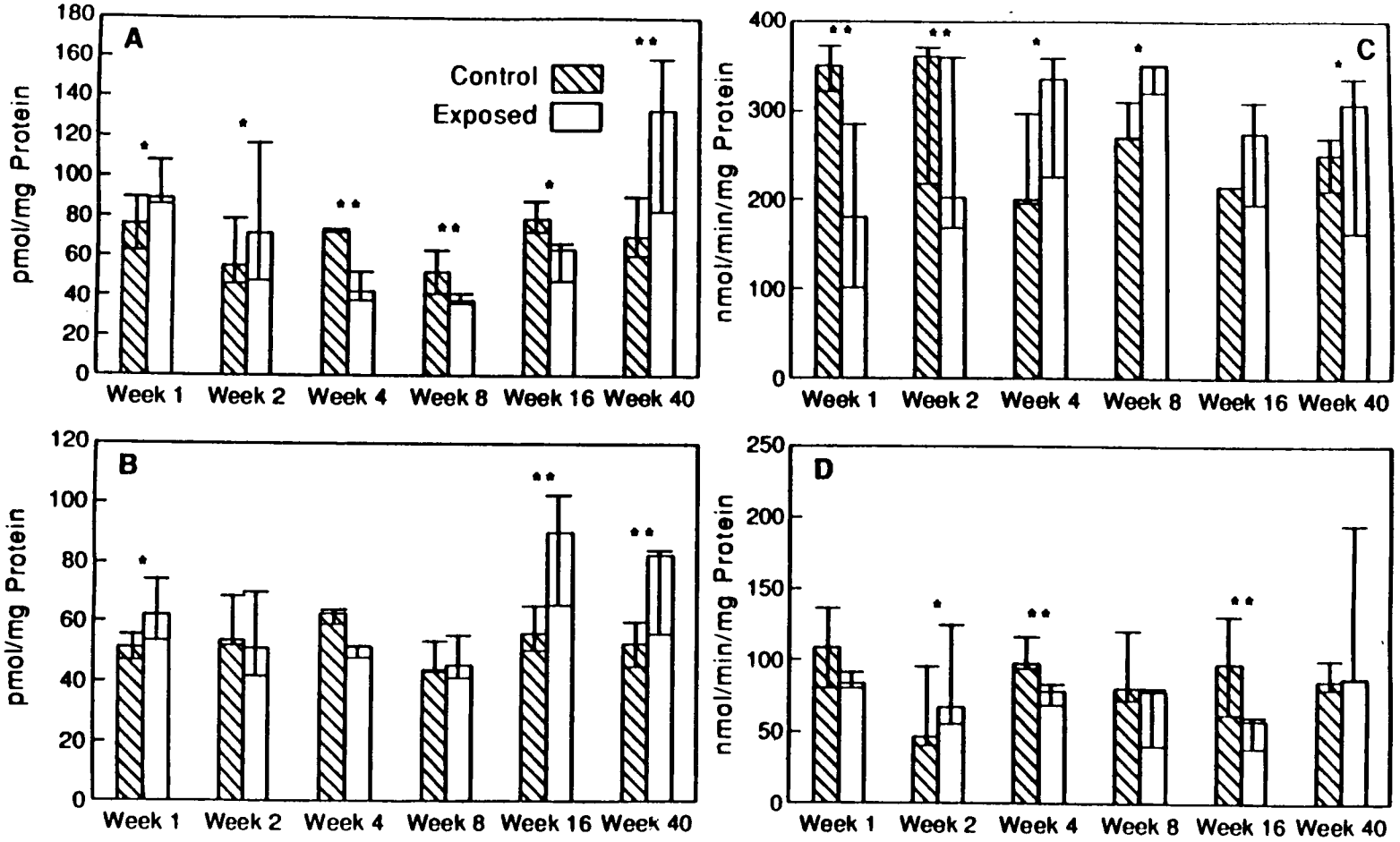
Figure 5 - Photograph of an agarose gel in which DNA from the livers of bluegill sunfish have been electrophoresed. The electrophoresis was run under denaturing conditions to differentiate between DNA samples with different numbers of strand breaks. The DNA was stained with ethidium bromide and photographed under UV light. "F-values" indicated above the lanes were obtained using the alkaline unwinding assay and are inversely proportional to the number of DNA stand breaks.

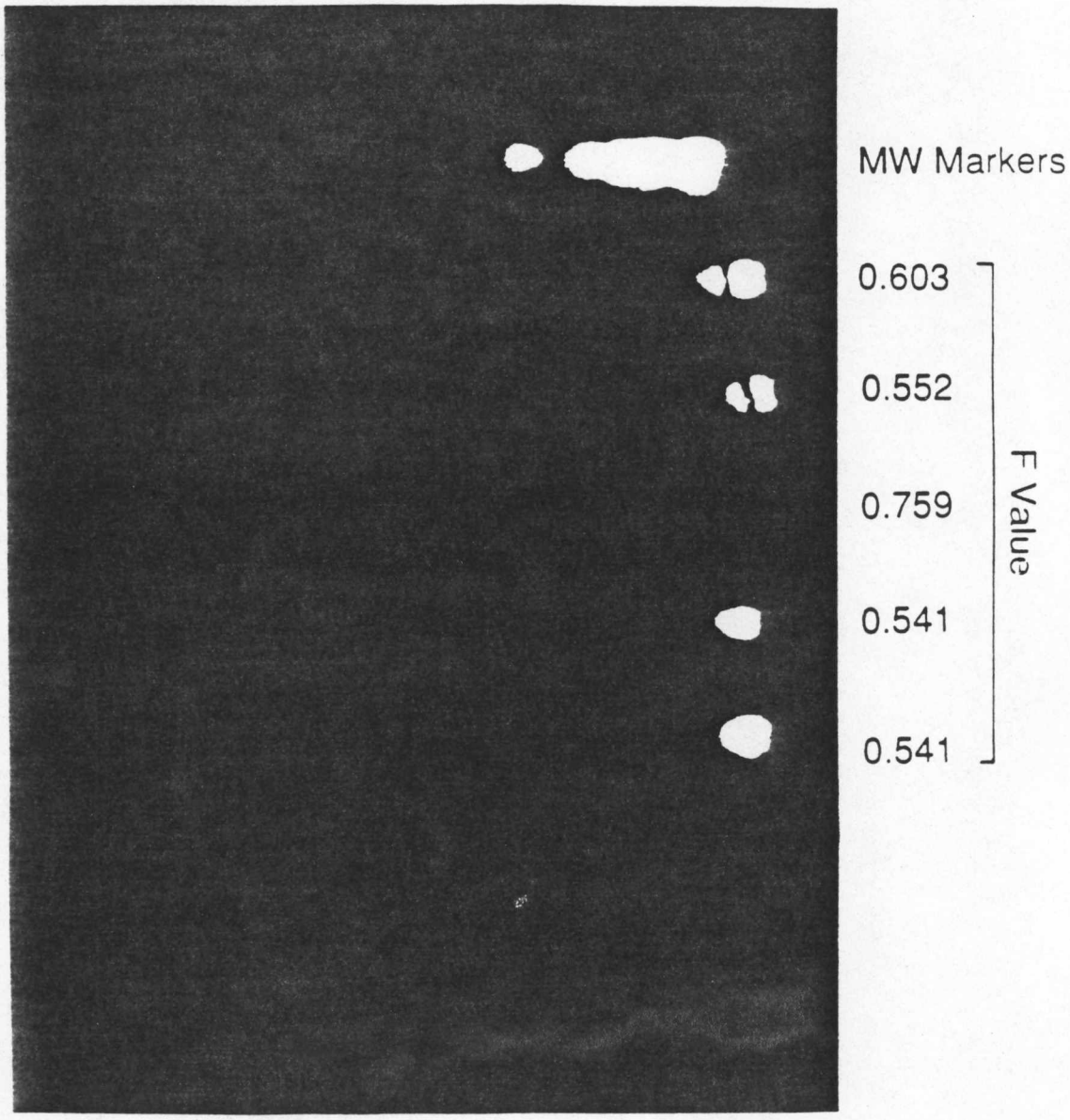
Figure 6 - ZPP content of the blood (A), hematocrits (B), spleen somatic indices (C), and kidney ATPase activities (D) of bluegill sunfish. Fish were exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars represent the range. * $P < 0.10$; ** $P < 0.05$.

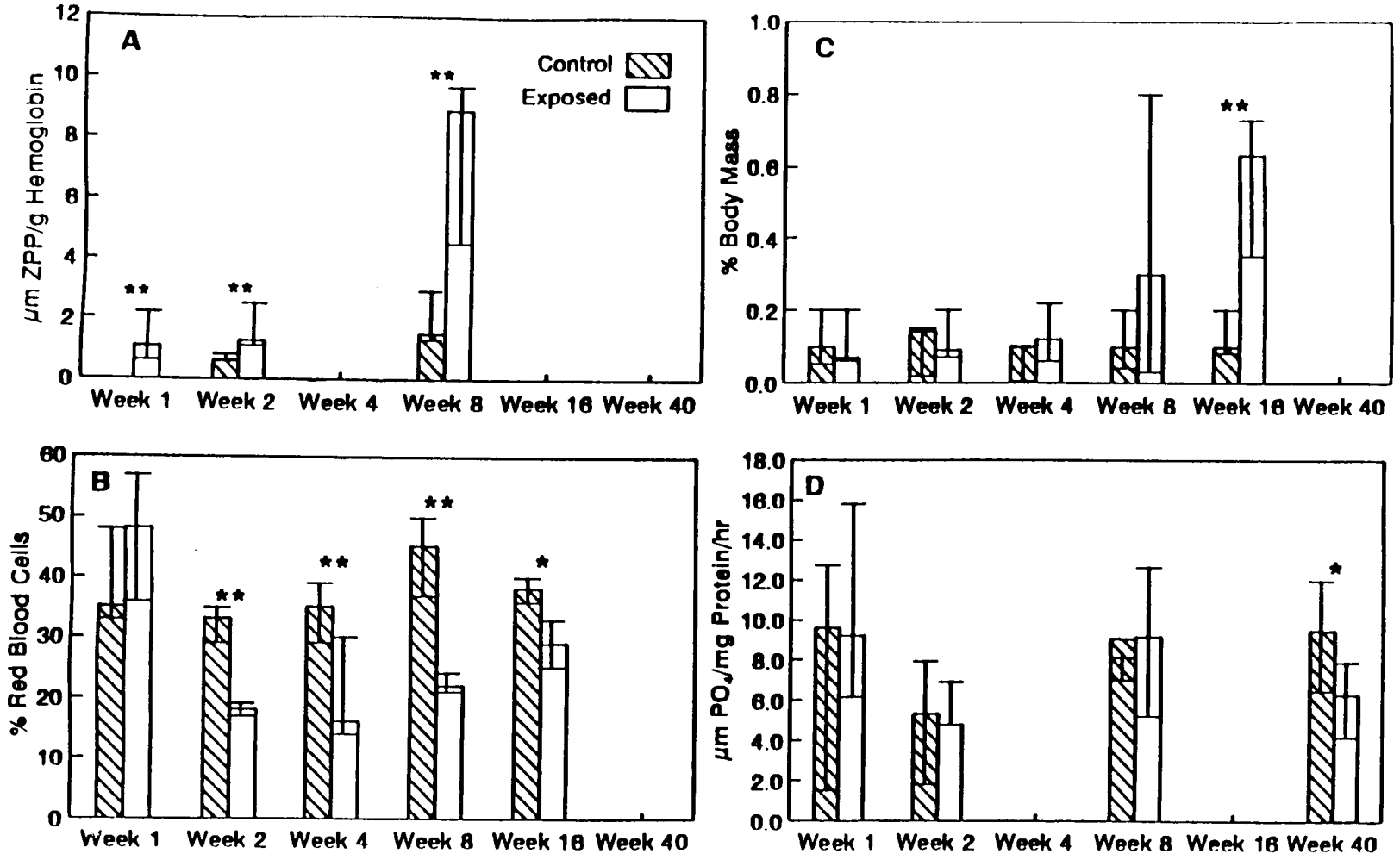

ORNL-DWG 92H-1746
A
B
Control
Exposed
μm PO4/hr/mg Protein
μm/hr/mg Protein
0
10
20
30
40
50
*
**
Week 1
Week 2
Week 8
Week 40


ORNL-DWG 92H-1747
A
Control
Exposed
μm PO4/hr/mg Protein
8
6
4
2
0
**
*
**
**
B
μm PO4/hr/mg Protein
6
5
4
3
2
1
0
*
**
**
**
Week 1
Week 2
Week 8
Week 16
Week 40


ORNL-DWG 92H-1749
A
Control
Exposed
pmol/min/mg Protein
70
60
50
40
30
20
10
0
*
**
**
**
**
**
B
% Body Mass
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40


A
Control
Exposed
pmol/mg Protein
180
160
140
120
100
80
60
40
20
0
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40
C
nmol/min/mg Protein
400
300
200
100
0
B
pmol/mg Protein
120
100
80
60
40
20
0
D
nmol/min/mg Protein
250
200
150
100
50
0


MW Markers
0.603
0.552
0.759
0.541
0.541
F Value


A
Control
Exposed
µm ZPP/g Hemoglobin
0
2
4
6
8
10
12
**
**
**
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40
C
% Body Mass
0.0
0.2
0.4
0.6
0.8
1.0
**
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40
B
% Red Blood Cells
0
10
20
30
40
50
60
**
**
**
*
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40
D
µm PO4/mg Protein/hr
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
*
Week 1
Week 2
Week 4
Week 8
Week 16
Week 40

# PART 3

# ASSESSING GENOTOXIC INSULT: DETECTION OF DNA STRAND BREAKS IN FISH BLOOD CELLS BY AGAROSE GEL ELECTROPHORESIS

## JUSTIFICATION

In the last chapter several biomarkers of genotoxic contamination were used. One of these was DNA strand breakage, and one method of measuring strand breakage was gel electrophoresis. Although this technique is routinely used to quantify strand breakage in radiobiology and carcinogenesis studies, its application to environmental monitoring has been limited.

Furthermore, in the last chapter the examination of genotoxic effects was primarily limited to the liver. However, it was suggested in the last chapter that blood may be used as a surrogate for more invasive techniques.

Therefore the objective of this chapter are to further pursue the use of electrophoresis in genetic ecotoxicology and the use of blood cells for detecting DNA strand breaks.

**ABSTRACT**

DNA, isolated from the blood cells of bluegill sunfish (Lepomis macrochirus) exposed in the laboratory to sediment containing genotoxic compounds (i.e., polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals), was examined for strand breakage by agarose gel electrophoresis. Prior to electrophoresis the blood cells were imbedded in agarose plugs and incubated with proteinase. After electrophoresis under either neutral (pH 7) or alkaline (pH 12) conditions the median molecular length (MML) of the DNA distributed in the gel was determined. These quantitative measures were used to estimate the difference in the number of double strand and single breaks between DNA preparations. Both types of strand breakage were found to be greater in fish exposed to sediment contaminated with genotoxic compounds as compared to non-exposed fish. A statistically significant correlation was demonstrated between the MML value obtained by the electrophoretic assay reported here and the F-value (measure of DNA double-strandedness) obtained by the alkaline unwinding assay.

## INTRODUCTION

Chemical or physical agents are considered genotoxic if they exert an adverse affect on the integrity (structure and/or function) of DNA. When an organism is exposed to genotoxins in their environment, determination of the effects on the integrity of its DNA can be assessed by observing changes at many different levels of biological complexity. Approaches for detecting changes to DNA integrity include assays that demonstrate nucleotide base modifications (McCarthy et al. 1991, Malins and Haimanot 1991, Shugart 1990, Dunn et al 1987, Shugart et al. 1987), DNA strand breakage (Shugart 1998), sister chromatid exchange or other chromosomal aberrations (Maddock et al. 1986, Dixon et al. 1982), unscheduled DNA synthesis (West et al. 1988), micronuclei formation (Hose et al. 1987, Das and Nanda 1986), point mutations (Yung-Jin et al. 1991, McMahon et al. 1990), and variations in nuclear DNA content (McBee and Bickham 1988). Of these approaches, the determination of DNA strand breakage is probably the least technically complex and/or time consuming because it does not involve extensive chemical or cytological preparation of the samples.

Studies in our laboratory on the effect of exposure to genotoxic agents have generally focused on structural damage to DNA (adducts and strand breaks) of the liver of fish or other aquatic organisms (Meyers-Schone et al. 1993, McCarthy

et al. 1991, Shugart 1990, Shugart 1988, Shugart et al. 1987). This is primarily because the liver is the major organ of xenobiotic detoxication (Buhler and Williams 1989), and thus is the primary site of procarcinogen activation (Varanasi et al. 1989). There are certain drawbacks, however, to using the liver as the source of DNA. For example, obtaining DNA from liver tissue is not only labor-intensive and time-consuming, it also involves sacrificing the animal, which can draw negative perceptions of environmental biomonitoring from certain aspects of society. Additionally, fish liver preparations contain endonucleases (probably because the pancreas in most Teleosts is closely associated with the liver [Harder 1975]) that degrade DNA unless quickly inactivated (personal observation).

An alternative approach is to examine structural damage to DNA in tissues which are more accessible, have low nuclease activities and can be obtained without sacrificing the animal. Because non-mammalian vertebrates posses nucleated red blood cells, fish blood is one such tissue that meets the above criteria and it also contains large amounts of DNA per unit volume (Curtis and Nochumson 1990). An additional advantage of using blood cells is that they can be easily embedded in agarose plugs for subsequent electrophoresis. DNA prepared in this manner has the advantage that it is not subject to shearing forces encountered in conventional DNA isolation

procedures (i.e., homogenization, pipeting, etc.) which can produce extraneous strand breakage.

Agarose gel electrophoresis is a technique that has been used for assessing DNA damage in yeast or cultured mammalian cells (Ehlers et al 1991, Wlodek et al 1991, and refs. therein), but its application in environmental toxicology has been limited (Theodorakis et al. 1992). Briefly, this method entails embedding cells in low melting agarose and digesting overnight with detergent and protease. The resulting, partially-purified DNA is then subjected to agarose gel electrophoresis. Migration of the DNA within the gel matrix is size dependent and detection is easily accomplished with ethidium bromide staining. By conducting the assay under neutral (pH 7) or alkaline (pH 12) conditions, detection of both double-strand and single breaks in the DNA is possible.

The goals of this work were two fold. The first was to evaluate the effectiveness of agarose gel electrophoresis as an analytical technique for the detection of DNA strand breaks using blood cells from fish as a source of DNA. The second was to compare data generated by this method for detecting strand breaks in DNA with the alkaline unwinding method (Shugart 1988). Several types of DNA damage in the liver, as well as a wide suite of biomarkers in other organs, have been previously characterized in the fish used in this study (Theodorakis et al. 1992). This study focuses on the DNA of blood cells, which includes both red and white blood cells.

However, the vast majority of nucleated blood cells in fish blood are composed of red blood cells, with the white blood cells only making up a very small percentage of the total blood cell population. Therefore, for the purposes of this paper, the contribution of white blood cells to the DNA pool was ignored.

## METHODS AND MATERIALS

### Experimental Design

Bluegill sunfish (Lepomis macrochirus) were exposed in the laboratory to sediment containing high levels of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals, and were fed tubificid worms which were also exposed to this sediment. Control fish were maintained in identical laboratory conditions, except that they were exposed to relatively non-contaminated sediment and fed non-contaminated worms. The blood samples used in this study were taken from fish exposed to control or contaminated sediment for 8 weeks. Details of the experimental design and sampling strategy are presented in Theodorakis et al. (1992). Blood was collected via caudal vein puncture into EDTA-treated vacuum tubes and stored at -120° C.

### Chemicals

Standard and low-melting agarose were obtained from Bio-Rad Corp. (Richmond, VA). Ultra-pure phenol/chloroform/isoamyl alcohol was obtained from BRL (Bethesda, MD). All other reagents, enzymes, and commercially prepared DNA (lambda

DNA standards and calf thymus DNA) were obtained from Sigma Chemical Co. (St. Louis, MO).

**Agarose Gel Electrophoresis**

Agarose Plug Preparation - Preparation of agarose plugs for electrophoresis was adapted from Ehlers et al. (1991). A small volume of blood (5 $\mu$l for the single strand break assay, 2 $\mu$l for the double strand break assay) were diluted in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0) to make 100 $\mu$l. The diluted cells were equilibrated to 37° C and mixed with an equal volume of pre-melted 1% low melting agarose also equilibrated to 37° C. The agarose plugs were cast by loading 15 $\mu$l of the cell suspension into the sample wells of a 2% standard agarose gel and chilling the gel at 4° C for 1 h. The plugs were cut from the gel, placed in digestion buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.5% sarcosyl, pH 7, with proteinase K at 1 mg/ml) and incubated overnight at 55° C. The plugs were chilled, loaded into a 1 or 0.6 % agarose gel (see below), and sealed into place with a small aliquot of low melting agarose (37° C) prior to electrophoresis. This technique resulted in approximately 0.40 $\mu$g DNA per well for the single-strand gel and 0.15 $\mu$g DNA per well for the double-strand gel.

DNA Single-strand Break Assay - The method of DNA-denaturing gel electrophoresis was adapted from Freeman et al. (1986). The 1% agarose gels (1g standard agarose, 50 mM NaCl, 4 mM EDTA, 100 ml total volume) with the plugs sealed into the wells were soaked for 1 h. in alkaline running buffer (30 mM NaOH, 2 mM EDTA, pH 12) to denature the DNA. Electrophoresis was then carried out at 7.5 V/cm for 1.5 h in alkaline running buffer. (CAUTION: Denaturing gel electrophoresis causes the alkaline running buffer to become very hot, which will ruin the gel if the buffer is not cooled. The simplest way to do this is to circulate the buffer through coiled tubing immersed in an ice bath.) The gels were then neutralized in 200 mM Tris base (pH 7) for 45 min, stained in an ethidium bromide solution (0.04 mg/ml) for 15 min and photographed.

DNA Double-strand Break Assay - The methods for this assay were adapted from Wlodek et al. (1991). The plugs were sealed into a 0.6% agarose gel containing 0.5 μg/ml ethidium bromide. TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) was used both to make the gel and as a running buffer. The electrophoresis was then run at 1.5 V/cm for 14 h, after which the gel was photographed.

**Alkaline Unwinding Assay**

DNA from blood cells was isolated by suspending a blood sample (approximately 20-100 μl) in 2 ml TE, adding 200 μl 10% sacrosyl and gently mixing the solution. This solution was extracted twice with phenol/chloroform/ isoamyl alcohol (25:24:1, v:v:v) and once with chloroform. The DNA was precipitated from the aqueous phase with ethanol, vacuum dried, and resuspended in TE. The alkaline unwinding assay was performed as described by Shugart (1988). Data are reported as "F-values", which are inversely proportional to the number of strand breaks.

**Calculation of Double-strand Breaks Using Alkaline vs. Neutral Electrophoresis**

In order to determine the relationship between the number of double-strand breaks as revealed by the alkaline and neutral pH electrophoresis methods, DNA which was relatively free of single-strand breaks was isolated using the techniques for the alkaline unwinding assay described above. Double-strand breaks were produced by passing the DNA solution through a 50 μl Hamilton syringe (22s gauge needle) 6 times. The DNA was then subjected to electrophoresis under alkaline and neutral conditions.

**Data Collection and Analysis**

The number of strand breaks was quantitated by calculating the median molecular length (MML) of the DNA in the sample. First, the negatives of the photographs were scanned with a scanning laser densitometer (Bio-Rad Corp., Richmond, VA) to produce a plot of absorbance vs. electrophoretic migration distance. The MML was calculated from the distance at which the area under the absorbance curve was divided into equal halves (Fig.1). A Hind III digestion of lambda phage DNA, consisting of various DNA fragments of known molecular length, was used to calculate the molecular lengths of the samples. These molecular weight were included in every gel alongside the samples of unknown molecular length. The relative number of strand breaks (RNSB) in the DNA of exposed fish above that of the DNA of control (unexposed) fish was calculated from the following formula (Freeman et al. 1986):

$$RNSB = 1/L_n \text{ (exposed)} - 1/L_n \text{ (control)}$$

where RNSB is the relative number of DNA strand breaks per kilobase and $L_n$ is the average molecular length (number of nucleotide bases/DNA molecule). The computation of $L_n$ from photographs of agarose gels involves complex integral formulas, but a good approximation can be achieved from the relation (Ehman and Lett 1973):

$$L_n = 0.6MML$$

where MML is the median molecular length described above.

For double-strand breaks, an alternative method for determining DNA damage was also employed. This involved calculating the ratio of the amount of DNA migrating out of the sample well to the amount of DNA remaining in the well (DNA migration ratio: DMR). Relative amounts of DNA were determined as the area under the absorbance curves for the two DNA populations (Fig. 2). In order to ensure that the absorbance curve from the densitometer tracing was linearly proportional to the amount of DNA in the agarose gel, known amounts of calf thymus DNA were subjected to electrophoresis. The area under the absorbance curve for each DNA concentration was measured from the densitometer tracing and a linear regression of area under the curve vs. DNA concentration was calculated (Statgraphics software, Orem, UT).

Differences between the MML data for control and exposed fish were tested using a t-test. To compare the electrophoretic assay with the alkaline unwinding assay, linear regression and correlation were calculated for a curve of MML data vs. F-values (Statgraphics software, Orem, UT).

## RESULTS

### Calculation of Median Molecular Length and Relative Number of Strand Breaks

For the single-strand break assay, the MML for control fish was found to be significantly greater than that for exposed fish (Table 1[2]). The patterns used to generate these data can be visualized in the photograph of the gels (Fig 3) and in a sample densitometer tracing of a gel (Fig 1), in which the DNA of the exposed fish is more "smeared" than that of the controls. Thus the DNA of exposed fish contains smaller DNA fragments, presumably as a result of genotoxin-induced strand breakage. Note that for the samples in lanes 2 and 3 of the alkaline gel (Fig 3), the DNA is present as non-uniform streaks rather than a uniform smeared distribution (eg. lanes 6-9). When this phenomenon occurred, those samples were not used for strand break calculations. A possible explanation for the aberrant streaking pattern is given in the discussion.

Using the formula for RNSB, it was calculated from the data in Table 10 that the exposed fish were found to have 10.1 more breaks per $10^5$ bases than control fish. I.e., for exposed fish $L_n$ = 0.6MML = 0.6 x 10.9 Kb = 6.5 Kb, while for

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[2]All tables and figures for Part 3 are found in the appendix to Part 3.

controls $L_n$ = 0.6 x 31.9 Kb = 19.1 Kb. Thus, RNSB = $6.5^{-1}$ - $19.1^{-1}$ = 0.101 breaks/Kb, or 10.1 breaks per $10^5$ bases. Similar data were calculated for the double-strand break assay (Table 1, Fig. 4), and exposed fish were found to have 2.2 more double-strand breaks per $10^5$ bases than controls (RNSB = $[0.6 \times 23.8]^{-1}$ - $[0.6 \times 35.0]^{-1}$ = 0.022 breaks/Kb). However, the calculated value of the MML is dependant on the amount of DNA loaded into the well (Fig. 5)

When performing electrophoresis under neutral conditions, much of the DNA is too large to migrate out of the sample well unless it is damaged (eg. see Figs. 2 and 4, eg. lanes 4 & 5). Thus Wlodek et al. (1991) suggested that the relative amount of DNA migrating out of the sample well is a better indication of double-strand breakage. Therefore the ratio of (the amount of DNA migrating out of the well) ÷ (the amount remaining in the well) was also calculated. This ratio (hereafter referred to as the DNA migration ratio, or DMR) was found to be greater for exposed fish than for controls (Table 1). It was also found that the area under the absorbance curve was linearly proportional to the amount of DNA loaded into the well (Fig. 6A), so this method seems to be a reliable method of relative DNA quantitation.

**Comparison of Electrophoretic and Alkaline Unwinding Assays**

In comparing the results of the alkaline electrophoretic assay with that of the alkaline unwinding assay, there was found to be a statistically significant correlation between MML value and F-values (Fig. 6B).

**Calculation of Double-strand Breaks Using Alkaline vs. Neutral Electrophoresis**

DNA which contained predominantly double-strand breaks was compared to DNA which was relatively free of strand breaks in order to compare the values of RNSB determined by the alkaline and neutral electrophoretic assays. It was found that number of double-strand breaks determined with the alkaline assay (5.3 breaks per $10^5$ bases) was roughly double that using the neutral Ph assay (2.5 breaks per $10^5$ bases).

## DISCUSSION

DNA strand breakage in not an uncommon occurrence in a cell. Heat energy causes thousands of abasic sites per cell per day which are rapidly repaired (Alberts et al. 1989). This is an example of an insult to DNA that indirectly results in strand breakage (i.e., the initial damage is a loss of a base from the DNA chain and repair results in a temporary gap in the DNA molecule). Ionizing radiation and free radicals can cause strand breakage directly, whereas other physical agents such as UV light and many chemical agents that are genotoxic potentiate alterations to the DNA molecules that are candidates for repair (eg, photoproducts, adducts, base modifications, etc.) and thus for the occurrence of strand breaks (Shugart et al. 1992).

In the study detailed in this paper agarose gel electrophoresis was determined to be an excellent analytical technique for detecting DNA strand breakage. In addition this is the first reported use of fish blood cells as a source of DNA for strand break analysis by this technique. Fish blood can be accurately measured and a given volume easily embedded in agarose plugs. This not only reduces the number of steps necessary to obtain DNA from the tissue, but also it protects the DNA from extraneous damage. DNA prepared in this manner is not subjected to the usual shearing forces encountered in conventional isolation procedures (i.e., homogenization,

pipeting, etc.). It can be argued that DNA damage in blood cells has no serious consequences to fish health. As noted above, an important aspect of this study is that these blood cells are from the same fish in which various types of genotoxic damage were found. Thus the presence of DNA damage in blood cells can suggest similar damage in other organs as well.

It should be noted that the alkaline electrophoretic assay reveals total strand breaks (both single- and double-strand) while the neutral pH assay only reveals double-strand breaks. In this study it was found that the number of double-strand breaks determined by the alkaline assay was roughly twice that using the neutral pH assay. Thus the number of single-strand breaks alone is equal to the number of total strand breaks minus (2 x the number of double-strand breaks). For the data collected in this experiment, the number of total strand breaks was 10.1 per $10^5$ bases, and the number of double-strand breaks was 2.2 per $10^5$ bases. Thus, the number of single strand breaks alone is 4.7 breaks per $10^5$ bases (10.1 - 2(2.2) = 4.7). The number of single-strand breaks is greater than the number of double-strand breaks because chemical genotoxins should produce predominantly single-strand breaks due to the removal of chemically altered nucleotides (Sancar and Sancar 1988). However, since double-strand breaks are repaired more slowly than single-strand breaks (Peak et

al. 1991) they probably have a tendency to accumulate more rapidly than single-strand breaks.

As Wlodek et al. (1991) found, determining the amount of DNA migrating out of the sample well may be a more accurate determination of double-strand breaks, since the MML can only be calculated for the DNA which migrates out of the well. Determining double-strand breakage is important, since not only are double-strand breaks more difficult to repair, they may have more dire consequences in terms of cytotoxicity or carcinogenesis (Peak et al. 1991, and refs. therein). When DNA is purified within agarose plugs, the amount of shearing during DNA isolation within the agarose plugs has been minimized. Thus the molecular size of the DNA molecule may be quite large, and thus may not migrate out of the sample well at all. Wlodek et al. (1991), however, used radioisotopes and liquid scintillation counting to determine the percent DNA migrating out of the well. In this study such use of radioactive materials was obviated by estimating relative amounts of DNA from the photographic negatives.

However, when using the electrophoretic technique, one should be aware that the amount of DNA loaded into the gel can affect the apparent MML (Fig. 5). This is because the amount of "smearing" that can be detected from the photographic negatives decreases with decreasing DNA concentrations. Thus, one should use caution to ensure that similar amounts of DNA are assayed when comparing different samples.

Another disadvantage to the use of agarose plugs is that frozen blood cells may clump together and cause "streaking" (Fig. 3, lanes 2 & 3) rather than a uniform band or smear (Fig. 3, lanes 6-9), and is a particular problem with frozen cell pellets. However, if the assay cannot be performed immediately after blood collection, the blood can be stored under sterile conditions at 4° C for at least 2 days without degradation of the DNA (personal observation). The digested plugs can also be stored at 4° C for at least 2 weeks without significant DNA degradation (Ehlers et al. 1991, Wlodek et al. 1991).

There are other techniques besides electrophoresis which can detect DNA damage in living organisms. These techniques will be reviewed below in order to compare them to the electrophoretic technique. A technique similar to the use of agarose plugs was developed by Singh et al. (1988). In their procedure, mammalian blood was suspended in low melting point agarose, which was coated on a microscope slide and digested with proteinase and detergent. The slide was subjected to electrophoresis and stained with ethidium bromide. DNA damage in the leukocytes was examined by microscopic image analysis to determine the amount of DNA remaining in the cell vs. that leaving the cell nucleus (Singh et al. 1988). There are three major drawbacks to this technique. One is that complex and expensive videographic and image analysis equipment needs to be used to obtain the data. Because fish blood has nucleated

red blood cells, the amount of DNA in a very small sample of fish blood can be visualized with the naked eye. Another drawback is that, for each sample, a large number of leukocytes (at least 50) need to be sampled in order to get a statistically relevant sample of the total leukocyte population (Singh et al. 1988). This increases data collection time. A third drawback is that direct quantitation of strand breaks is not possible.

There are also other methods of determining DNA strand breakage, such as alkaline unwinding (Shugart 1988), alkaline elution (Kohn 1976), alkaline sucrose gradient centrifugation (Veatch and Okada 1969), and DNA precipitation (Olive 1988). The alkaline unwinding assay subjects DNA samples to heat and high pH to partially separate the strands. A fluorescent DNA dye is then added, which binds preferentially to double-stranded DNA. The fluorescence of the partially denatured DNA is compared to that of non-denatured DNA to estimate the proportion of single-stranded DNA, which is used as and index of strand breakage. The theory is that DNA with more strand breaks will unwind faster under alkaline conditions, so more DNA will be in the single-stranded form (Rydberg 1975). A similar technique is the alkaline elution technique. In this technique, DNA is bound to a polyvinyl chloride filter membrane. An alkaline solution is then applied to the filter, which preferentially binds double-stranded DNA. Thus when the DNA is partially denatured by the alkali, the single stranded

DNA is eluted while the double-stranded DNA remains on the filter. The relative proportion of single-stranded to double-stranded DNA is estimated by measuring the amount of DNA in the eluate and on the filter (Kohn et al. 1976). Another technique which uses alkali to denature DNA is alkaline sucrose gradient centrifugation (Veatch and Okada 1969). In this technique, isolated DNA is applied to an alkaline sucrose gradient and centrifuged at 35,000 rpm for 2 h. The centrifuge tube is punctured at the bottom, the DNA is collected in 20-25 fractions, and the amount of DNA in each fraction is determined. From these data it is possible to calculate the relative number of DNA molecules per unit mass. The theory is that when DNA contains strand breaks there are more DNA molecules per unit mass (Veatch and Okada 1969). One assay that does not use alkaline condition is the DNA precipitation assay. In this assay, proteins and associated DNA are precipitated with sodium dodececyl sulfate and KCl. If there are strand breaks, however, some of the DNA does not precipitate with the proteins. The amount of strand breakage is directly proportional to the ratio of the amount of DNA in solution: the amount in the precipitate (Olive 1988). One major drawback to many of these assays is that they are laborious and time consuming. In many cases, the use of the electrophoretic technique required much less man-hours to complete.

In our laboratory we are most familiar with the alkaline unwinding assay, so the discussion below will focus mainly on comparing this assay with the electrophoretic assay. However, several of the points made below may also be applicable to the alkaline elution and DNA precipitation assays.

Besides minimizing DNA damage during sample processing the electrophoretic technique uses less DNA than the alkaline unwinding assay. Although the DNA detection limits for a fluorimeter (used in the unwinding assay) and ethidium bromide (used in electrophoresis) are comparable (personal observation), to obtain a single data point for the alkaline unwinding assay you actually have to perform 3 separate assays. This may become an important consideration when the amount of sample available is limiting. In addition, because the reproducability of the alkaline unwinding assay relies on precise additions of small amounts of reagents, it is more prone to pipeting errors and operator bias than the electrophoretic assay.

Another advantage of the electrophoretic assay over the alkaline unwinding, elution or precipitation assays is that the latter assays determine total strand breakage, thus cannot distinguish between double- and single-strand breaks. In addition, although the latter assays can produce comparative data (Shugart 1988), the electrophoretic assay can produce more quantitative data in terms of differences in the actual number of strand breaks. The equations used to determine this

were derived from the alkaline sucrose gradient centrifugation technique (Freeman et al. 1986). Like the electrophoretic technique, the centrifugation technique can be used to calculate actual numbers of strand breaks. Also, the centrifugation technique can distinguish between double- and single-strand breaks. However, this technique requires centrifuging the DNA on a sucrose gradient, collecting up to 25 fractions of centrifuged DNA per sample, and determining the amount of DNA in each fraction. This can become quite laborious if large numbers of samples are analyzed. For the electrophoretic assay, on the other hand, up to 30 samples can be run on the same gel, reducing the time required for analyzing large numbers of samples.

A further advantage of the electrophoretic assay is that the DNA from the gels can be blotted onto nylon or nitrocellulose membranes and probed with specific genes (Sambrook et al. 1989). For example, we are currently formulating experiments using oncogene probes to see if fish from contaminated areas posses DNA strand breaks within known oncogene coding regions.

In conclusion, it has been found that agarose gel electrophoresis with agarose plugs is able to detect an increase in both double- and single strand DNA breakage in fish exposed to genotoxic compounds. This assay has several advantages over other strand breakage assays. For example, it can be used to distinguish between double- and single-strand

breaks and double strand breaks, and can produce more quantitative data than other strand break assays. Thus assaying DNA stand breakage using electrophoresis with fish blood cells can be used effectively in biomonitoring programs and environmental genotoxicity assays.

## ACKNOWLEDGEMENTS

This project was sponsored in part by the Oak Ridge Y-12 Plant, Department of Environmental Management, Health, Safety, Environment and Accountability Division and by an appointment to the U.S. Department of Energy Laboratory Cooperative Postgraduate Research Training Program administered by the Oak ridge Associated Universities. This project was also supported in part by the U.S. Army Biomedical Research and Development Laboratory (DOE project No. 1061-BO47-A1). The Oak Ridge National Laboratory is administered by Martin Marietta Energy Systems Inc. under contract DE-AC05-A4OR21400 with the U.S. Department of Energy.

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Table 1. Median molecular length (MML) and DNA migration ratio (DMR) from fish exposed to control and genotoxin-contaminated sediments. Data were generated from agarose gel electrophoresis of blood DNA under alkaline or neutral conditions.

| | Alkaline | Nuetral | |
|---|---|---|---|
| Treatment | MML* | MML* | DMR† |
| Control | 31.9 ± 7.8 | 35.0 ± 3.7 | 0.85 ± 0.30 |
| Contaminated | 10.9 ± 10.7 | 23.8 ± 11.5 | 13.31 ± 17.6 |
| P-Value† | 0.001 | 0.02 | 0.02 |

*The median molecular length is expressed in kilobases (Kb, thousands of nucleotide bases).

**The DNA migration ratio is calculated as the amount of DNA migrating out of the sample well ÷ the amount of DNA remaining in the sample well of the agarose gel.

§Student's t-test. $H_o$: No difference between treatments.

**Figure Legends**

Figure 1 - Sample densitometer tracing taken from a photographic negative of DNA bands in an alkaline (pH 12) agarose gel, subjected to electrophoresis. The curve depicts measurement of optical density (O.D.) vs. distance along the photographic negative of the gel, and represents the distribution of DNA in one of the lanes of the gel. The median molecular length (MML) is calculated as the length of a DNA molecule which would migrate to a point that divides the absorbance curve into two equal halves. MML values are depicted for DNA from blood of bluegill sunfish exposed to genotoxic (A) and non-genotoxic (control, B) sediments.

Figure 2 - Sample densitometer tracing taken from a photographic negative of DNA bands in a neutral (pH 7) agarose gel, subjected to electrophoresis. The curve depicts measurement of optical density (O.D.) vs. distance along the photographic negative of the gel, and represents the distribution of DNA in one of the lanes of the gel. A) DNA from the blood of bluegill sunfish exposed to genotoxic sediment. B) DNA from the blood of bluegill sunfish exposed to non-genotoxic (control) sediment. Notice that in the exposed fish more of the DNA has migrated out of the well, while for controls more or the DNA remains in the sample well.

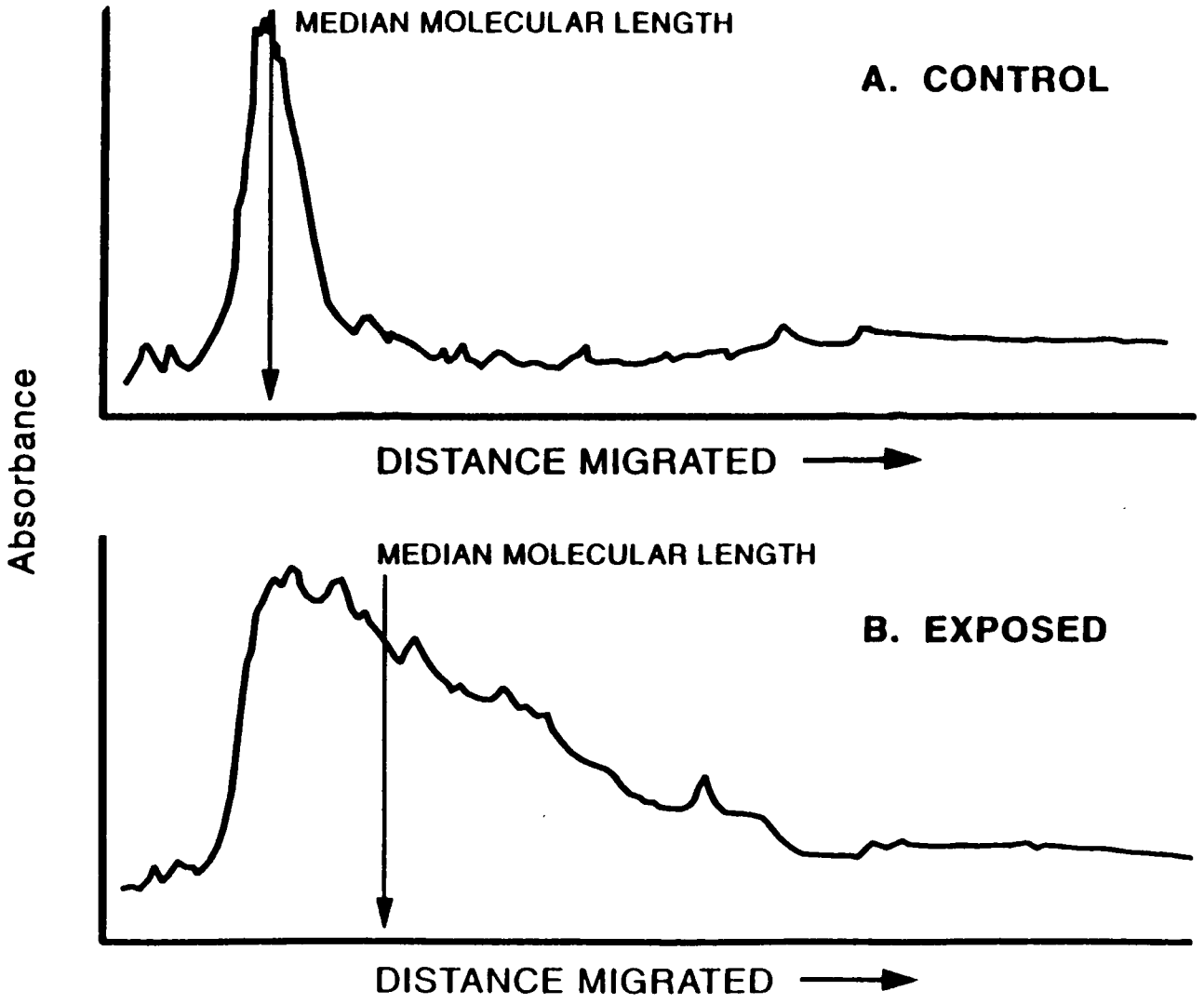
Figure 3 - Photograph of an agarose gel run under alkaline (pH 12) conditions and stained with ethidium bromide. Lanes 1-3 and 6-9 contain DNA from the blood of bluegill sunfish exposed to genotoxic sediments for 8 weeks. Lanes 4, 5 and 10-15 contain DNA from fish exposed to non-genotoxic sediments (control fish) for 8 weeks. DNA bands on either side of the samples are molecular weight markers (Hind III digests of Lambda phage DNA).

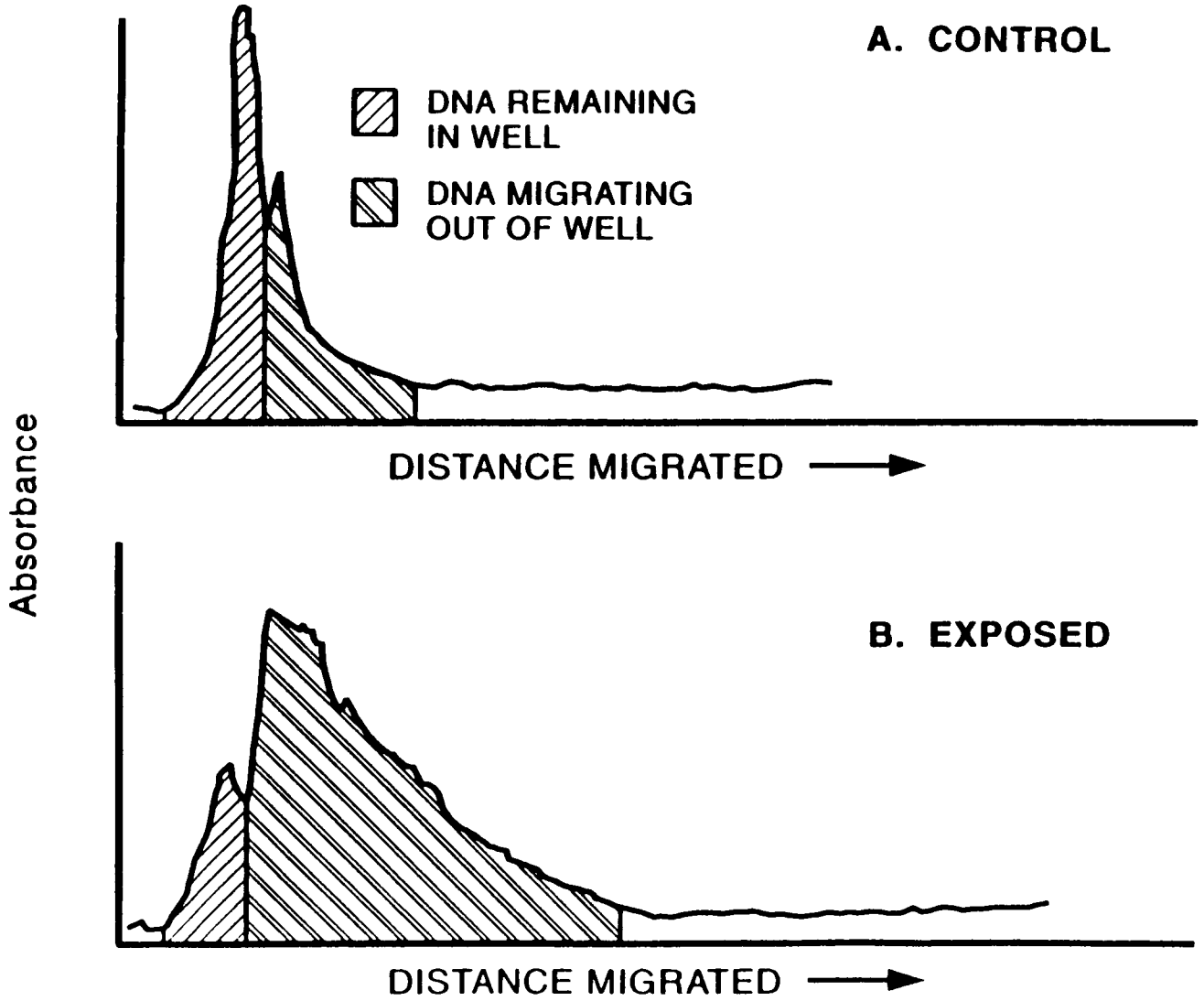
Figure 4 - An agarose gel run under neutral (pH 7) conditions, stained with ethidium bromide, and photographed under UV light. Lanes 1-3 and 6-9 contain DNA from the blood cells of bluegill sunfish exposed to genotoxic sediments. Lanes 4,5, and 10-14 contain DNA from fish exposed to non-genotoxic sediments (control fish). Note that in control fish much of the DNA does not leave the sample well (eg. lanes 4 & 5). The sample wells can be observed as faint squares near the top of the gel. DNA bands on either side of the samples are molecular weight markers (Hind III digests of Lambda phage DNA).

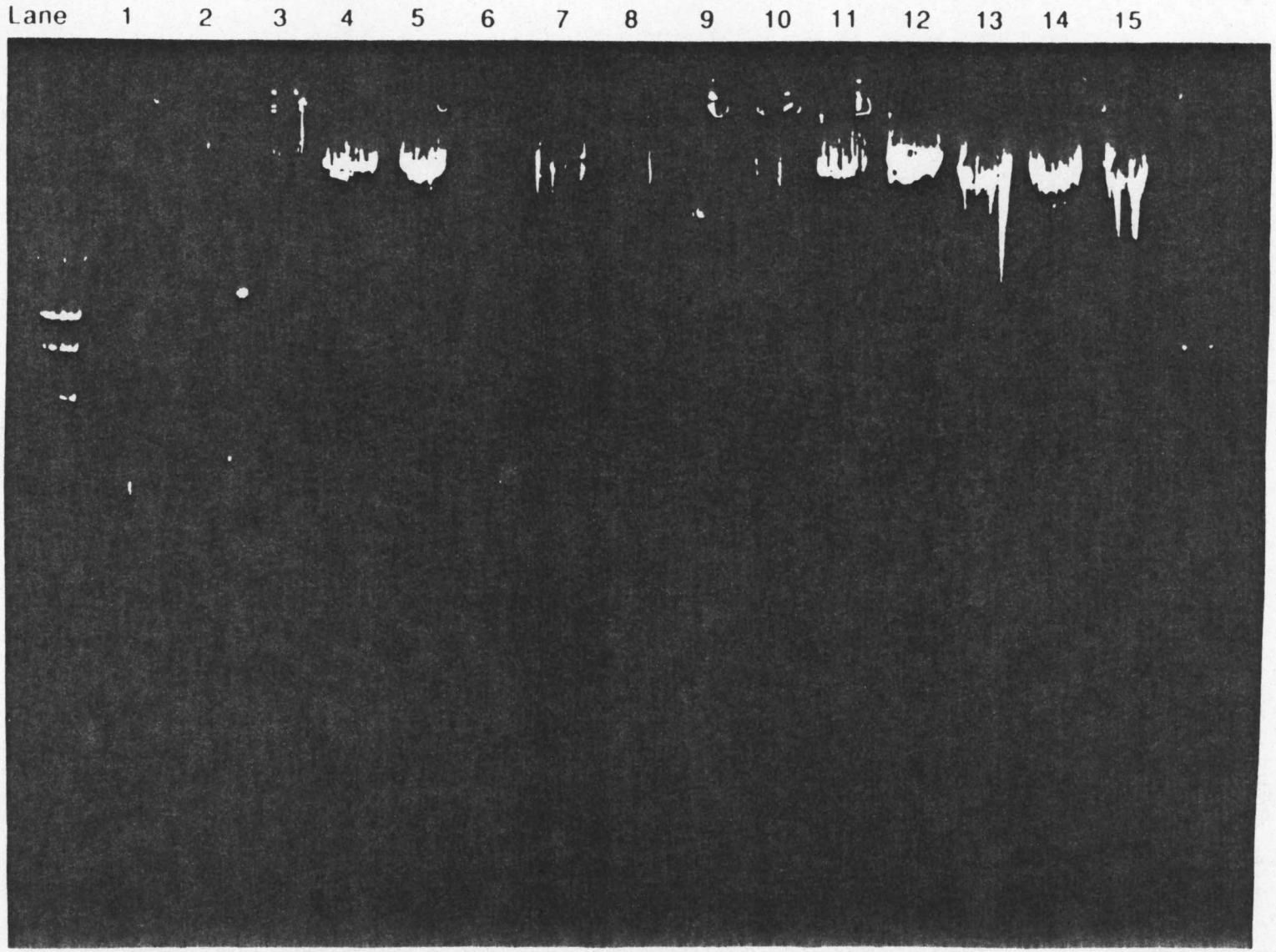
Figure 5 - An agarose gel with known amounts of DNA, stained with ethidium bromide, and photographed under UV light. The numbers above the lanes indicate how much DNA was loaded into each lane and the apparent median molecular weight of the DNA in each lane. The median molecular weight was calculated from

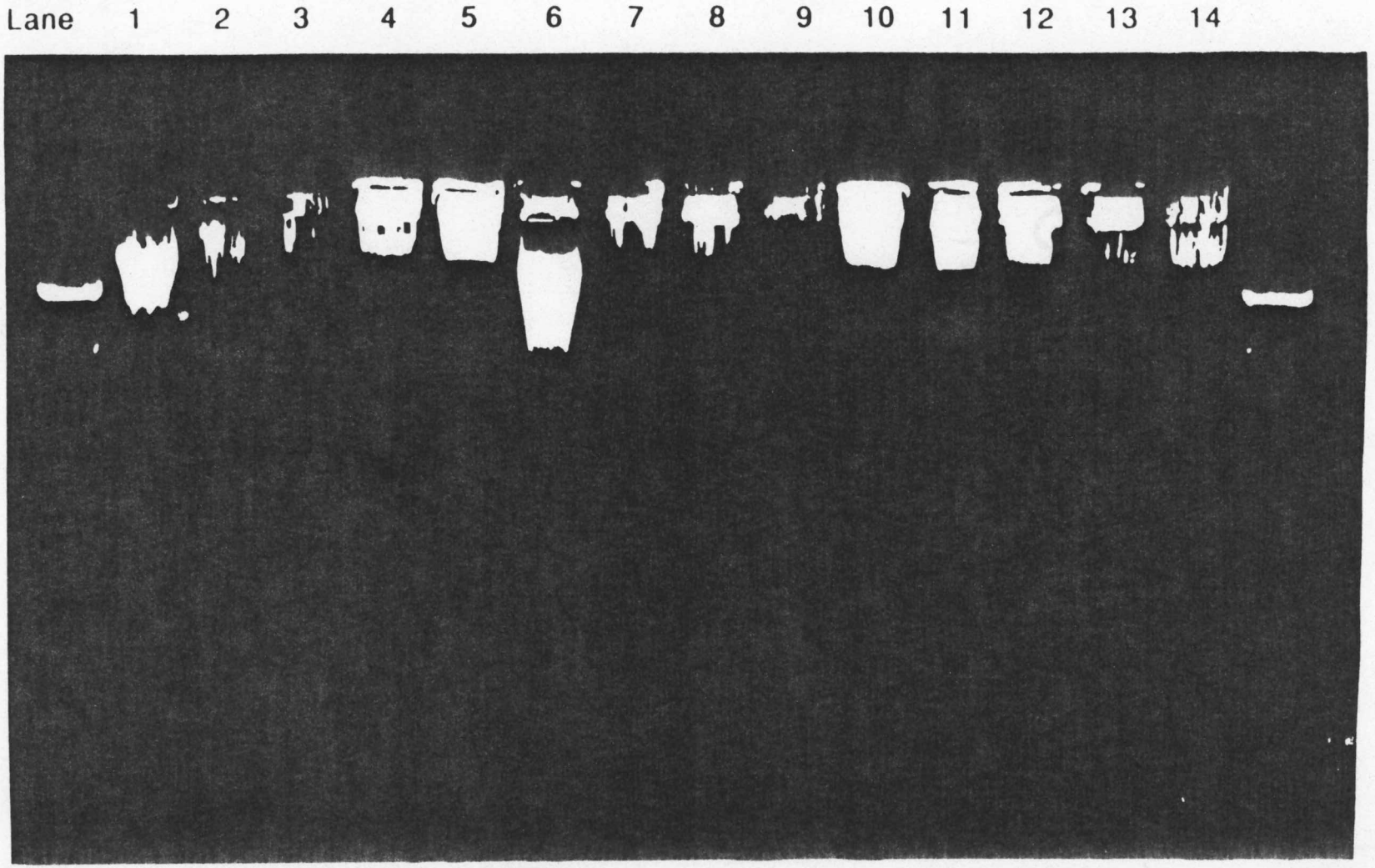
the distribution of the DNA in the gel compared to molecular length markers if known length. All lanes contain DNA from the same sample.

Figure 6 - Linear regressions of parameters for electrophoretic determination of DNA strand breakage. A) Linear regression of the amount of DNA loaded into a sample well of an agarose gel vs. the area under the densitometer absorbance curve. The densitometer absorbance curve was measured from a photographic negative of an agarose gel stained with ethidium bromide and represents absorbance of the negative film vs. distance. B) Linear regression of f-values (determined by the alkaline unwinding assay) vs. the median molecular length (determined electrophoretically) of the DNA from the fish used in this study.

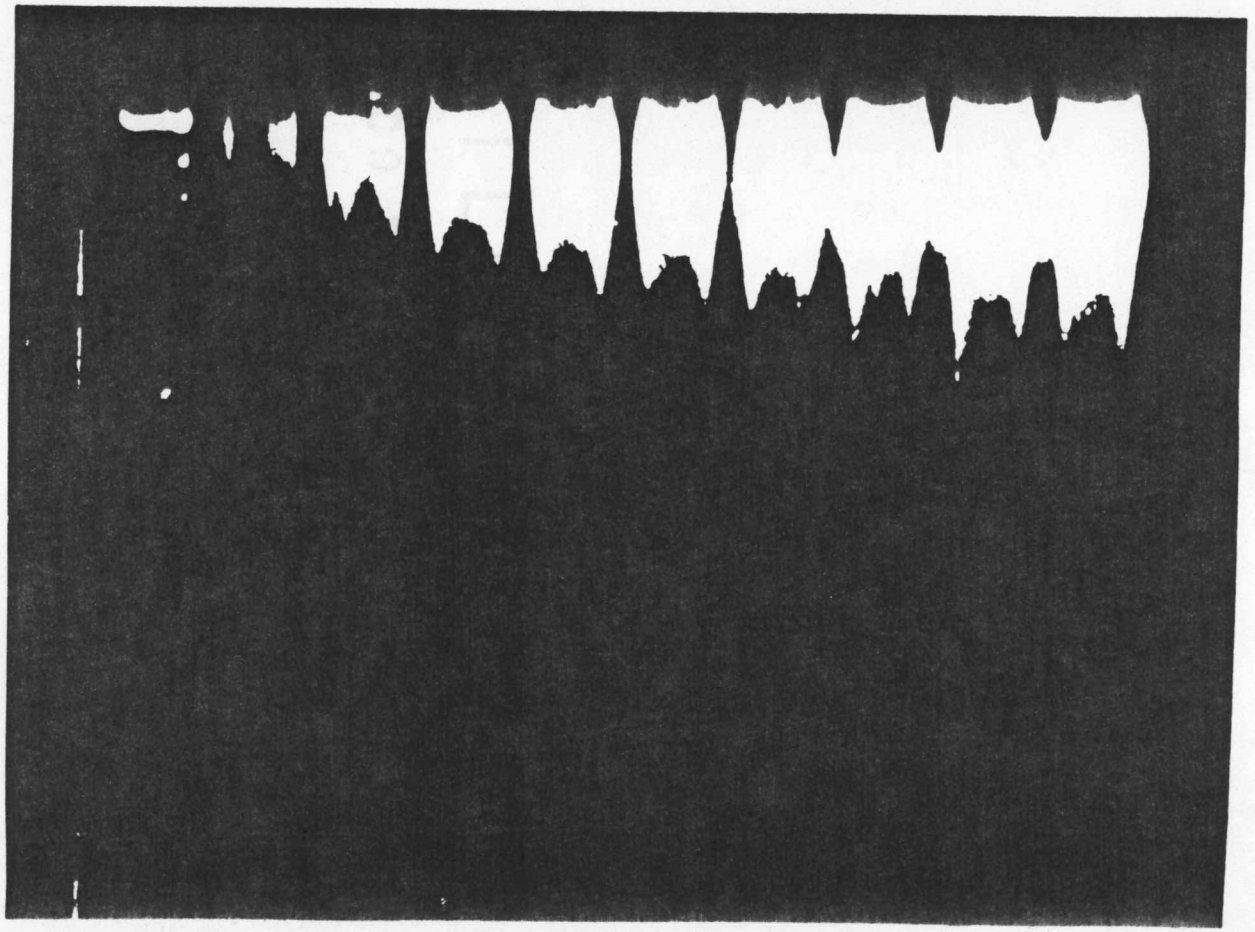

MEDIAN MOLECULAR LENGTH
A. CONTROL
Absorbance
DISTANCE MIGRATED
MEDIAN MOLECULAR LENGTH
B. EXPOSED
DISTANCE MIGRATED

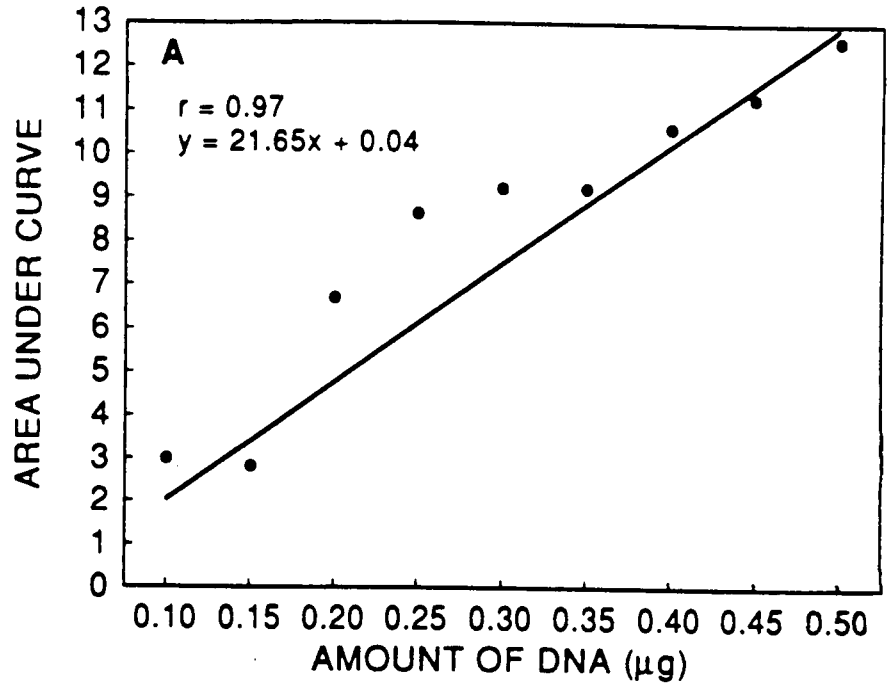

A. CONTROL
DNA REMAINING IN WELL
DNA MIGRATING OUT OF WELL
Absorbance
DISTANCE MIGRATED
B. EXPOSED
DISTANCE MIGRATED

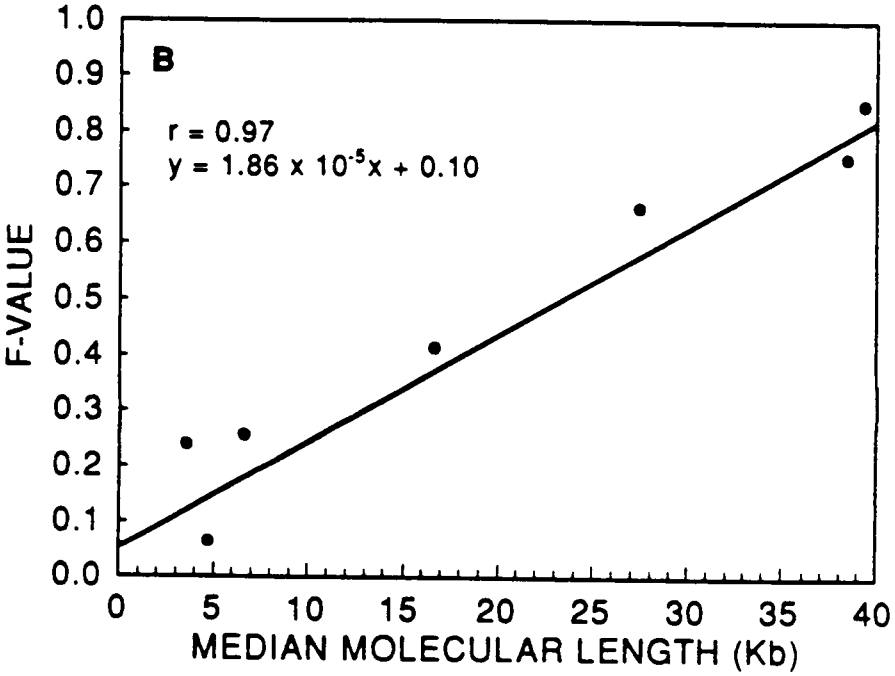

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

| Amount of DNA (µg): | 0.10 | 0.15 | 0.20 | 0.25 | 0.30 | 0.35 | 0.40 | 0.45 | 0.50 |
|---|---|---|---|---|---|---|---|---|---|
| Median Molecular Length (kb): | 8.6 | 7.7 | 7.1 | 7.2 | 6.7 | 6.2 | 6.1 | 5.7 | 4.9 |




A
r = 0.97
y = 21.65x + 0.04
AREA UNDER CURVE
0 1 2 3 4 5 6 7 8 9 10 11 12 13
0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50
AMOUNT OF DNA (µg)


B
r = 0.97
y = 1.86 x 10-5x + 0.10
F-VALUE
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
0 5 10 15 20 25 30 35 40
MEDIAN MOLECULAR LENGTH (Kb)

# PART 4

# ECOTOXICOLOGY OF RADIONUCLIDES IN MOSQUITOFISH I: DNA STRAND BREAKS AND THEIR RELATIONSHIP TO FECUNDITY

## JUSTIFICATION

In the last two parts gel electrophoresis was used to examine DNA strand breakage. In part 2, strand breakage was assessed for liver DNA in part 2 and blood DNA in part 3. However, neither of these was a comparative study examining both tissues. It was suggested in part 3 that blood may be used as a non-destructive biomarker, but in order for it to be defensibly used in routine monitoring the relationship between responses of blood and other organs must be known. Therefore one of the purposes of this Part is to compare DNA damage in blood and liver tissues.

In addition, the studies in parts 2 and 3 focused on the use of electrophoresis in laboratory studies, and this technique had yet to be used on field samples. Therefore another purpose of this study was to verify its use in environmental analysis with a field study.

Finally, this section was part of larger study examining the effects of radionuclides on population-level responses. In part 2 it was suggested that variation in response may lead to selection for resistant variants and eventually altered population genetic patterns. This line of investigation was undertaken in three major sections: 1) Examination of strand breaks in radionuclide-contaminated populations and determination of the relationship between strand breaks and fecundity. This relationship was of interest because it has

implications both to evolutionary processes and higher-level ecological responses (eg, population and community dynamics) 2) Examination of population genetic responses in contaminated populations and 3) examination of the relationship between strand breaks and genotype.

**ABSTRACT**

Mosquitofish (Gambusia affinis) were collected from Pond 3513, a small pond that is contaminated with $^{137}Cs$, $^{90}Sr$ and other radionuclides, and from White Oak Lake, a settling basin contaminated with radionuclides and chemical genotoxins. Both sites are located on the Oak Ridge Reservation. Fish from non-contaminated populations were also collected. DNA was isolated from both liver and blood cells and examined for DNA total- (both single and double) and double-strand breaks alone by gel electrophoresis. In general, total- and double-strand DNA breaks were slightly more prevalent in fish from radionuclide-contaminated sites than from uncontaminated sites. In addition, there were differences in the number of strand breaks between liver and blood cells. Also, fecundity and number of malformed embryos were determined in fish from all sites. It was found that, for fish from the contaminated sites, the number of DNA strand breaks was negatively correlated with fecundity, and that females with malformed embryos in their broods had slightly more DNA strand breaks than did females with no malformed embryos. These findings have implications for both ecological risk assessment and evolutionary ecology.

## INTRODUCTION

The multitude of commercial and industrial activities in which humans engage result in the release of numerous xenobiotic compounds into the environment. One such class of compounds include genotoxicants: substances which chemically or physically alter the structure and function of DNA in a detrimental way. Chemical genotoxic agents include compounds such as polycyclic aromatic hydrocarbons which modify the structure of nucleotide bases (Phillips and Simms 1979). Chemical genotoxins are released into the environment by a multitude of processes, including burning of fossil fuels, production of industrial waste and urban runoff (McElroy et al. 1989). Physical genotoxic agents include ionizing radiation, which primarily damage DNA through the production of free radicals (Roots and Okada 1975). Anthropogenic sources of ionizing radiation include radionuclides which are released into the environment as the result of production and use of radioisotopes, nuclear energy, and nuclear weapons (NAS 1980).

When one examines DNA damage produced by genotoxic agents, there are many methods used to assay such damage. One of the simplest methods is to determine DNA strand breaks. Chemical agents produce strand breaks when the modified nucleotides are enzymatically removed from the DNA molecules by the cell's DNA repair mechanisms, or when such modified bases are "bypassed" during DNA replication (Hoeijmakers 1993,

Sancar and Sancar 1988). Ionizing radiation can produce strand breaks in one of two ways. First, one of the atoms in the DNA molecule can be converted into a free radical by radioactive particles or the secondary radicals they produce (Roots and Okada 1975, Stanton et al 1993, Shutle-Frohlinde and Von Sonntag 1985). Such a DNA radical is unstable, and quickly leads to a strand break. Alternatively, ionizing radiation can induce single-stranded nicks in the DNA through endonuclease activity (Katcher and Wallace 1983, Bressler et al. 1979), which may be a response to nucleotide base damage (Shulte-Frohlinde and Von Sonntag 1985. While genotoxic chemicals primarily produce single-strand breakage (Hoeijmakers 1993, Katcher and Wallace 1983), ionizing radiation may produce both single- and double-stranded DNA breaks (Kirsch et al. 1991). This is because the genotoxic free radicals are concentrated in clusters around the particle tracks (Roots and Okada 1975), which may lead to clusters of DNA damage (Goodhead 1989, Ward 1988) and increase the likelihood of double-strand breaks.

The type and number of DNA strand breaks depends not only upon the type and dose of genotoxic agent, but also upon the organ in which strand breaks are examined. For example, the liver possesses an efficient DNA repair mechanism (Williams 1982), but has a relatively low rate of cell turnover (Arias et al. 1982). In contrast, DNA in fish red blood cells is repaired very slowly, if at all, because it is

transcriptionally inactive. However, fish blood cells have a high rate of turnover (Mahajan and Dheer 1980). Thus one would expect that the number of DNA strand breaks would be different in liver and blood cells when an organism is exposed to a genotoxic agent.

When DNA is damaged by a genotoxic agent, the results of such an insult may impair physiological functions. In terms of ecological risk assessment, such an impairment is significant only if it alters a parameter of ecological importance (Suter 1990). A physiological function which has ecological implications is reproductive performance, of which fecundity is one such measure. Alterations in fecundity could have ramifications both to population dynamics and population genetic structure. Although exposure to genotoxic agents has been shown to affect fertility of laboratory rats (Rattus norvegicus, McLachlan et al. 1981), and fish (Egami and Hama-Furikawa 1980, Kono 1980, Purdom and Woodhead 1973, Woodhead 1977), there is little information regarding the effect of genotoxicity on fecundity in an ecological setting (NCRP 1991, Woodhead 1984, Blaylock and Trabalka 1978).

Therefore the objectives of this study are to 1) determine if exposure to environmental radiation significantly higher than background results in an increase in DNA strand breakage; 2) determine if the amount of DNA strand breakage differs between blood and liver tissue; and 3) examine the relationship between the amount of strand breakage and

fecundity in the mosquitofish, Gambusia affinis. Radionuclide contamination was chosen as the genotoxic agent because such contamination is well documented and characterized on the Oak Ridge Reservation, which is operated by the Oak Ridge National Laboratory (Blaylock et al. 1993, Blaylock et al. 1991, Tamura et al. 1977). Mosquitofish were chosen as the organism for study because they are small, easily captured, and are livebearing fish, so fecundity and embryo abnormalities can be easily determined. Also the biology of this fish is well known and the responses mosquitofish populations to radionuclide contamination on the reservation have been well documented (Blaylock and Frank 1980, Blaylock and Trabalka 1978, Trabalka and Allen 1977).

It is hypothesized that radionuclide exposure will result in an increase in both singe- and double-strand breaks, that liver DNA will have fewer strand breaks than blood DNA, and individuals with a greater number of strand breaks will have a lower fecundity.

## METHODS AND MATERIALS

### Study Sites

Radionuclide-contaminated sites studied include White Oak Lake and Pond 3513, which are currently, or have been, part of the Oak Ridge National Laboratory radioactive waste disposal system. White Oak Lake, which is located on the Oak Ridge Reservation, has served as a final settling basin for low-level radioactive effluents from the Oak Ridge national Laboratory since 1943 (Blaylock et al 1991). Mosquitofish which inhabit this 17.9 ha lake are exposed to external radiation from radionuclides in water and sediment and internal radiation from the ingestion of radionuclides via the foodchain. Based on measurements with thermoluminescent dosimeters and dose calculations the radiation dose received by mosquitofish inhabiting White oak lake was estimated to be 43.8 rads/year at the sediment surface in 1975; however, dose rates in the 1960's conceivably should have been an order of magnitude greater (Trabalka and Allen 1977). In addition to radionuclide, White Oak Lake is also contaminated with organic chemicals and heavy metals which are known to be genotoxic or carcinogenic (Table 1, Blaylock et al. 1993).

Pond 3513 is a 0.45 ha man-made pond which received liquid waste from a low-level waste plant from 1944 until the mid 1970's. In 1977, an inventory of the radioactivity in the

pond indicated that the sediment contained 5.0 Ci of $^{239,240}Pu$, 200 Ci of $^{37}Cs$, and 33 Ci of $^{90}Sr$ (Tamura et al. 1977). The average radiation doses measured by thermoluminescent dosimeters exposed for two weeks at three stations in the pond measured at the water surface, at mid-depth, and at the surface of the sediments were 37,394, and 1150 rads/year, respectively. In July 1977, approximately 250 mosquitofish were introduced into this pond from Crystal Springs (see below). These fish have been exposed to levels of radiation significantly greater than background for more than 26 years (Blaylock and Frank, 1980).

The reference sites include Crystal Springs, a first order stream originating from an underground spring in Oak Ridge, TN, and Wolf Creek, a first order stream approximately near Oak Ridge. Neither of these sites have any known history of radionuclide contamination.

**Specimen Collection**

Adult female mosquitofish ranging in size from 30-45 mm were collected by seine or dipnet from each of the sites. For strand-break analysis, 28 fish were used from each site. The fish were transported back to the laboratory were they were anesthetized in a solution of 0.50 g/L tricane methanosulfonate (MS 222). The caudal peduncle was then severed at the anal fin and the fish were placed in fish

physiological saline (FPS: 6.44 mg NaCl, 11 mg Kcl, 22 mg $CaCl_2$, 12 mg $MgSO_4$, 7 mg $KH_2PO_4$, and 10 mg $NaHCO_3$ per L distilled water) plus 25 mM EDTA in order to collect red blood cells. The blood cells were washed once in FPS and frozen in liquid nitrogen. The livers and embryos were then removed, and the embryos were counted and scored for abnormalities. All tissues were then frozen in liquid nitrogen and stored at -70° C until analysis.

**Strand Break Analysis**

Liver or blood cells were homogenized in 300 µl FPS plus 25 mM EDTA. Thirty µl of 10% sarcosyl (sodium laurel sarcosine) was then added and the samples were allowed to sit on ice 10 min. The homogenate was then extracted with phenol:chloroform (1:1) and then chloroform. The DNA in the extract was precipitated with ethanol, vacuum dried and resuspended in TE (10 mM Tris buffer, 1 mM EDTA, pH 8.0).

Total DNA strand breaks (both single- and double strand breaks) were determined by agarose gel electrophoresis under alkaline (pH 12) conditions. Double strand breaks were determined by electrophoresis under neutral conditions. For alkaline gel electrophoresis, 500 ng of DNA was run on a BioRad wide mini-subcell apparatus with 30-well comb. For neutral gel electrophoresis, 200 ng of DNA was analyzed on the same apparatus. Detailed methods for electrophoresis are

described in part 3 of this dissertation. Gels were stained with ethidium bromide and photographed under UV light. The photographic negatives of the gels were analyzed with a densitometer (BioRad Corp., Richmond, VA) to produce an absorbance curve of absorbance vs. distance DNA migrated in the gel. Median molecular length (MML) of each DNA sample was then calculated from absorbance vs. distance curve (see part 3). When DNA is isolated from cells, the sample is composed of DNA molecules of various sizes. The MML represents the midpoint of the size distribution of the DNA molecules. The MML is inversely proportional to the number of strand breaks: when there are more strand breaks, the DNA is broken into smaller-sized fragments, so the MML will be lower.

**Statistics**

Because the numerical distributions of strand breaks were skewed, nonparametric test were used to test differences between populations, tissues, and seasons (Wilcoxon Rank-Sum test) and to test dependance relationships (Kendall test of correlation; Hollander and Wolfe 1973).

## RESULTS

### Comparisons Between Populations

The median molecular length of DNA of fish living in Pond 3513 was significantly lower than that from Crystal Springs or Wolf Creek (Fig. 1[3]), indicating an increased number of strand breaks in Pond 3513. This was true for both total- and double-strand breaks in the liver and blood. In general the same was true for White Oak Lake, with the exception that there is no difference between White Oak Lake and Crystal Springs for total DNA strand breaks in the liver (Fig. 1). The amount of total strand breaks in the blood was found to be greater in White Oak Lake than in Pond 3513, but the opposite was true for liver DNA. The amount of double-strand breaks was the same for these two sites in both tissues (Fig. 1). When the two reference sites, Crystal Springs and Wolf Creek, were compared it was determined that Crystal Spring had more total- and double-strand breaks (lower MML) than Wolf Creek (Fig. 1).

### Comparisons Between Tissues

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[3]All figures and tables for Part 4 are found in the appendix to Part 4.

The MML of DNA from blood and liver were also compared. For the White Oak Lake population, blood DNA had more total strand breaks than that of liver. This was true for both total ($p=0.05$) and double strand breaks ($p=0.04$, Wilcoxon rank-sum test). For Pond 3513, the liver had more total ($p<0.01$) and less double-strand DNA breaks ($p<0.01$, Wilcoxon rank-sum test) than did the blood (Fig 1).

**Comparisons Between Strand Breakage and Reproduction**

In comparing the degree of strand breakage of fish with and without abnormal embryos present in the brood, it was found that there was a trend toward females with abnormal embryos having a lower MML than fish without abnormals, but this was only statistically significant for double-strand breaks in blood DNA (Fig. 2). When fecundity of these females was compared to MML values, there was a positive correlation between the fecundity and MML for both types of strand breaks and tissues ($p<0.05$, Kendall test of correlation; Fig. 3).

**Comparisons Between Seasons**

All of the above data were calculated from fish collected in late June or early July 1992. However, there were some fish collected from early to mid may, 1992. The MML of fish from Pond 3513 was found to be significantly higher in fish

collected during the summer than in fish collected during the spring, although this is only statistically significant for total strand breaks in the blood and double strand breaks in the liver (Fig. 4). Additionally, the differences in fecundity between the sites depended on season. In the spring, the average fecundity of fish from Crystal Springs was greater than either of the two contaminated sites (Fig. 5). In the summer, the fecundity increased for all sites, but no generalizations can be made in terms of differences between contaminated and non-contaminated sites.

**Statistical Considerations**

It can be seen in some cases that there are statistically significant differences even though the differences in the medians may be small (eg., see Fig. 1B, White Oak Lake liver vs. blood MML. This difference is significant at 0.04). It should be noted that skewness of the distribution can affect the results of a nonparametric test: For example, if two samples are skewed in different directions the overall test may be statistically significant even though the medians are similar. This indicates that the differences are due to overall numerical distribution of the samples rather than simply differences in the medians.

# DISCUSSION

## Comparisons Between Populations

Results from this study indicate that mosquitofish collected from radionuclide-contaminated populations generally had more DNA strand breaks than did non-contaminated populations. This is to be expected, because there are numerous laboratory studies which show that animals or cultured animal cells exposed to gamma or X-rays exhibit strand breakage (eg, Ward 1988, Lett et al. 1977, Rydberg 1975, Anhström and Erixon 1973, Veach and Okada 1969). However, to our knowledge this is one of the first studies which show DNA strand breakage as the result of environmental radionuclide contamination in natural fish populations.

One explanation for the differences between White Oak Lake and Pond 3513 is that there are different types of contaminants in the two sites: White Oak Lake contains genotoxic chemicals - which primarily produce single-strand breaks - as well as radionuclides. Like White Oak Lake, Pond 3513 has elevated levels of $^{137}Cs$, $^{90}Sr$, and $^{60}Co$, but 3513 also is contaminated with plutonium isotopes. Unlike the aforementioned radionuclides, plutonium produces alpha particles as well. Alpha radiation has more energy and produces proportionately more double-strand breaks than does lower-energy radiation (Kampf and Eichhorn 1983).

Furthermore, Pond 3513 has a much higher level of radionulcide contamination than does White Oak Lake, so the fish in Pond 3513 are receiving a much higher dose rate. It is therefore not unreasonable to suggest that differences in contaminant type and amounts may lead to differences in the patterns of strand breakage.

**Comparisons Between Tissues**

The differences seen between the amount of strand breaks in the liver and the blood cells is probably due to differences in physiology between these two tissues. For the White Oak Lake population the blood seems to contain more strand breaks than does the liver. This is to be expected, because fish red blood cells are transcriptionally inactive (Mahajan and Dheer 1980), so they probably do not have an efficient repair mechanism, if any at all. This could have ramifications for environmental monitoring, because the liver is typically the organ of choice for monitoring DNA strand breakage (eg. Shugart 1988). However, if an organism has an efficient DNA repair mechanism, strand breakage may not be evident in the liver. However, even if strand breakage is not evident, this does not mean that exposure to genotoxins does not affect the organism. It may not be strand breakage per se that is deleterious to the organism, but rather repair of such breakage. For example, point mutations can occur during

repair of DNA strand breaks (Thilly and Call 1986, Freidberg 1985). Thus, monitoring genotoxin exposure in a tissue - such as blood - that has low DNA repair capacity may circumvent this problem. An exception to the finding that blood cells have more strand breaks than liver cells is found in Pond 3513 fish. Here liver cells have more strand breaks than do blood cells. Obviously there must be some other factor influencing DNA damage - perhaps tissue-specific bioaccumulation or increased red blood cell turnover. Further investigation is needed to clarify these findings.

**Comparisons Between Strand Breakage and Reproduction**

Another aspect of this study was to examine the potential effect of strand breakage on fecundity in radionuclide-contaminated populations. It was found that, within Pond 3513, there was an inverse correlation between fecundity and strand breakage; i.e., fish with fewer strand breaks were more fecund. A possible explanation is that genotoxicants, especially radiation, seem to have the greatest lethal effect on rapidly dividing cells (Thilly and Call 1986). Thus, strand breakage may interfere with gametogenesis or embryogenesis since both these processes involve rapid rates of mitosis. Note that the correlation between liver strand breaks and fecundity is stronger for double than for total-strand breaks. Double-strand breaks are a more serious lesion

in terms of cell killing (Kampf and Eicchorn 1983), mutagenesis and clastogenesis (Kraft et al. 1989), and are more difficult to repair than single-strand breaks (Peak et al. 1991, Ward 1988). Thus it would seem that double-strand breaks may have a more dire consequence on gametogenesis and embryogenesis than do single strand breaks.

Although exposure to genotoxicants has been shown to decrease fertility in laboratory animals (see Introduction), evidence that it also occurs in environmentally relevant species is scarce. In other studies it was found that the roach (Rutilis rutilis) living in radionulcide-contaminated lakes in Russia had lower fecundity than fish living in reference sites (Peshkov et al. 1978, Voronina et al. 1974, as reviewed in NCRP 1991). However neither of these studies have tried correlate degree of strand breakage with fecundity. In contradiction to these findings, past studies have found that mosquitofish from White Oak Lake had higher fecundity compared to a reference site (Trabalka and Allen 1977, Blaylock and Frank 1980), although it has been suggested that this may be an artifact (Trabalka and Allen 1977). In the present study, it was found that fecundity of spring populations was lower in contaminated sites than in a reference site, but for summer populations fecundity varies from site to site in manner that cannot entirely be explained by genotoxicity alone. Obviously there are other ecological factors which affect fecundity.

The fact that strand breakage seems to have a negative correlation with fecundity has ramifications for both environmental risk assessment and evolutionary biology. In terms of risk assessment, this provides a direct link between molecular and population effects of pollution. This is significant because molecular effects are easily and quickly determined, compared to ecological effects. In addition, molecular responses may give advanced notice of impending ecological perturbations, because molecular responses may appear before substantial ecological damage (usually apparent only after prolonged insult) can occur. However, the relationship between the two levels of effect has been unclear. Other studies have shown an indirect linkage between molecular and ecological effects of exposure to environmental contaminants (Adams et al. 1989), and the results presented here provide additional evidence which could directly associate these two levels of effect. In particular, if exposure to genotoxicants has an adverse effect on fecundity, this in turn could have an effect on such ecological parameters as recruitment, population growth and dynamics, productivity, and even community structure.

In terms of evolutionary biology, the correlation between fecundity and strand breaks suggests that resistance to or repair of strand breakage contains a fitness component. This would imply that individuals with certain genotypes have a selective advantage over others in the population when

challenged with genotoxicants. Thus exposure to genotoxins could affect population genetic structure because the genotypes which are more resistant to strand breakage would come to dominate the population.

A related finding is that females with abnormal embryos had more DNA strand breaks than females with no abnormal embryos. This has two possible explanations. One is that resistance to strand breakage is heritable: females which are less resistant to strand breakage produce offspring which are less resistant. Thus it may be that embryos which are more adept at resisting or repairing strand breaks are less likely to develop abnormalities. At present, however, the heritability of strand breaks and the relationship between abnormalities and strand breaks is unknown and warrants further study. A second explanation is that some females may be exposed to more external radiation or accumulate more radionuclides than others. Because mosquitofish are livebearers, the radiation dose to which females are exposed should be correlated to the dose received by their broods. I.e., females which receive a greater dose should have more DNA strand breakage and more abnormal embryos. As of yet, it remains to be seen if differential exposure or radionuclide accumulation between individuals actually occurs in contaminated populations, but these possibilities merit further investigation because they have important implications for environmental monitoring

## Comparisons Between Seasons

When comparing the number of strand breaks from fish collected in the spring vs. the summer, it is interesting to note that there was more DNA breakage in fish collected in the spring than in those collected in the summer. Such variables such as water temperature or food supply may influence the effect of genotoxicants on the degree of strand breakage. Another possibility is that the fish collected in the spring were all born during the previous year, but fish collected in the summer may include fish which were born in the spring. Thus the average age of fish, as well as the total amount of exposure time may differ between seasons. Such considerations may influence the degree of strand breakage. When collecting specimens for environmental monitoring of genotoxin exposure, variables such as water temperature and season require special attention. Additionally, formulation of ecological risk assessment programs must take into account the potential exacerbation of genotoxic damage earlier in the season.

## Statistical Considerations

One cautionary note that deserves consideration is that even though the correlations and comparisons in this are statistically significant, these differences and correlation coefficients are often small. At present, it is not known if

small differences such as these are of biological significance, but this point deserves further consideration.

**Conclusions**

In conclusion, it has been found that 1) environmental exposure to radionuclide contamination can lead to DNA strand breakage significantly higher than background; the relative amount of DNA damage in contaminated populations may be influenced by season 2) the relative amounts of DNA damage can differ between blood and liver tissue; the amount of strand breakage in the blood relative to the liver may depend upon the type and amount of contamination present, and 3) there was a correlation between strand breakage and fecundity, and there may also be a relationship between strand breakage in the mother and abnormalities in the offspring. These findings have implications for ecological risk assessment (providing one possible link between molecular and population responses) and implications for evolutionary biology (suggesting that strand breakage carries a fitness component).

**Future Directions**

The results of this study indicate that there can be many environmental variables which affect the number of DNA strand breaks and fecundity. One aspect which is missing from this

study is long-term laboratory exposures where genetic and environmental variables could be more rigidly controlled. Although logistical constraints have prevented such endeavors in the past, such studies are being planned for the future. Other avenues of future research include: 1) elucidation of the environmental variables which affect strand breakage, fecundity, and the relationship between the two; 2) examination of the relationship between fecundity and strand breaks induced by chemical genotoxins (as opposed to radionuclides); 3) investigation of the degree of heritability of susceptibility to strand breakage and evolution of resistance to genotoxicants and 4) determination of the effects of genotoxicant exposure on ecological parameters such as population dynamics and genetic structure, community dynamics, and productivity. Results from a preliminary study have suggested that population genetic structure is affected by exposure to radionuclides (see next section).

## ACKNOWLEDGEMENTS

This project was sponsored in part by the Oak Ridge Y-12 Plant, Department of Environmental Management, Health, Safety, Environment and Accountability Division and by an appointment to the U.S. Department of Energy Laboratory Cooperative Postgraduate Research Training Program administered by the Oak ridge Associated Universities. This project was also supported in part by the U.S. Army Biomedical Research and Development Laboratory (DOE project No. 1061-BO47-A1). The Oak Ridge National Laboratory is administered by Martin Marietta Energy Systems Inc. under contract DE-AC05-A4OR21400 with the U.S. Department of Energy.

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Table 1 - Concentrations (mg/kg) of selected contaminants found in White Oak Lake which are known to be genotoxic or carcinogenic*.

| Inorganic Contaminants | | Organic Contaminants | |
|---|---|---|---|
| Contaminant | Concentration | Contaminant | Concentration |
| Arsenic | 8.25 | Benzo[a]pyrene | 2.66 |
| Cadmium | 1.62 | Chrysene | 2.66 |
| Chromium | 183.00 | Dibenz[a,h]-anthracene | 2.66 |
| Lead | 40.25 | Flouranthrene | 2.64 |
| Nickel | 26.25 | Phenantrene | 2.61 |
| | | Pyrene | 2.58 |
| | | Phthalate Esters | 10.39 |
| | | Total PCBs | 1.98 |

*Source: Blaylock, B.G., M.L. Frank, L.A. Hook, F.O. Hoffman and C.J. Ford. 1993. White Oak Creek Embayment Site Characterization and Contaminant Screening Analysis. ORNL/ER-81. Oak Ridge National Laboratory, Oak Ridge, TN.

**Figure Legends**

Figure 1 - Median molecular lengths (MML) of DNA of mosquitofish from radionuclide contaminated (Pond 3513 and White Oak Lake) and non-contaminated populations. Molecular length is inversely proportional to the number of strand breaks. The data are presented as medians with first and third quartiles indicated by error bars. Data are presented for both total and double strand breaks for both liver and blood tissues. Bars labeled with similar uppercase (liver) or lowercase (blood) letters are not significantly different ($P<0.05$, Kruskall-Wallace test).

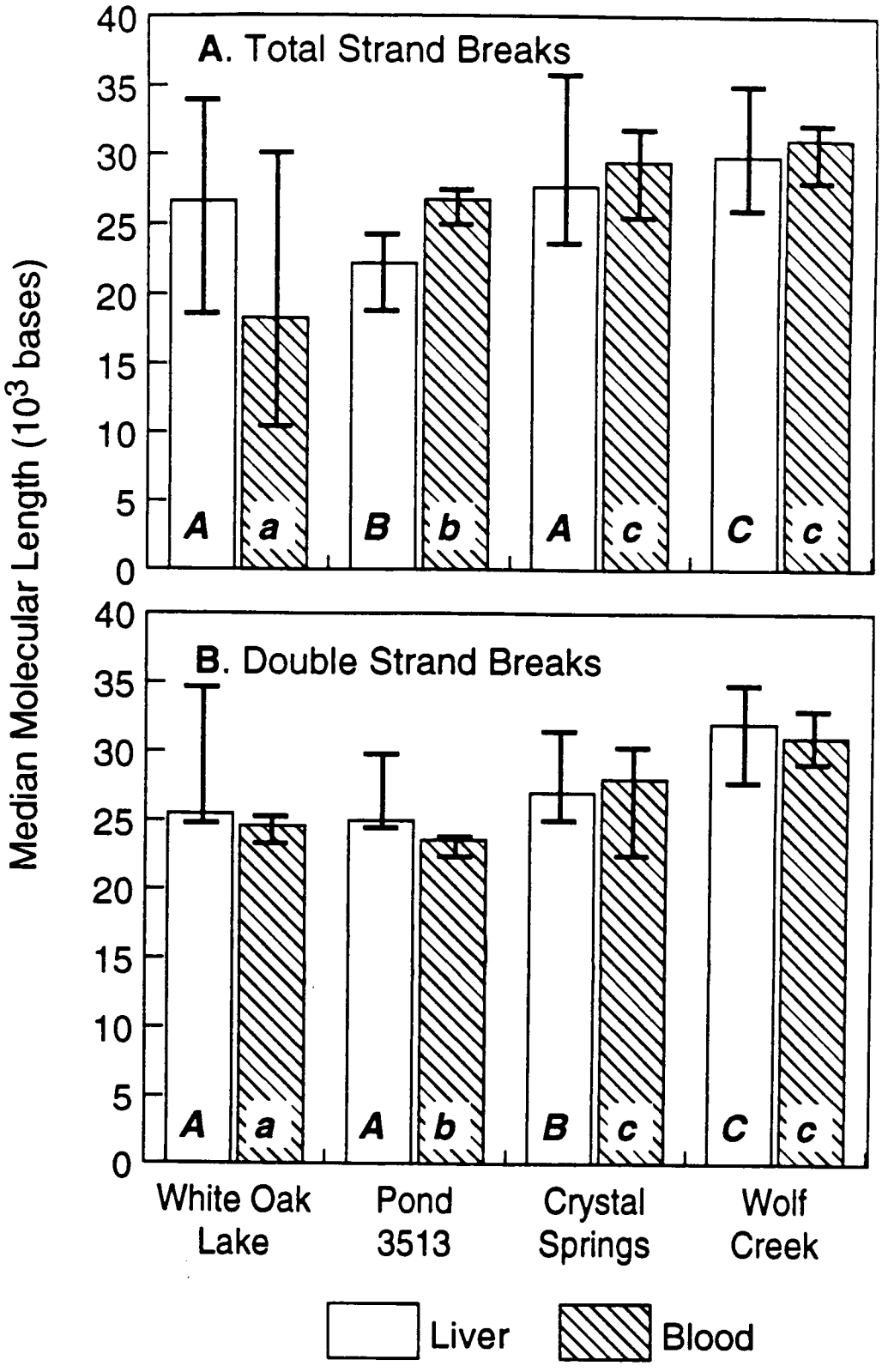
Figure 2. Median molecular length of DNA from mosquitofish collected from a radionuclide-contaminated pond (Pond 3513), both for fish with and without abnormal embryos in their brood. Data are presented for liver and blood tissues and for total and double-strand DNA breaks, and represent median values with first and third quartiles depicted by error bars. Numbers above each pair of bars denote probability values for the test of $H_o$: no difference between females with normal and abnormal embryos (Wilcoxon rank-sum test).

Figure 3 - Correlation between median molecular length (inversely proportional to strand breakage) and fecundity of mosquitofish collected from a radionulcide-contaminated pond

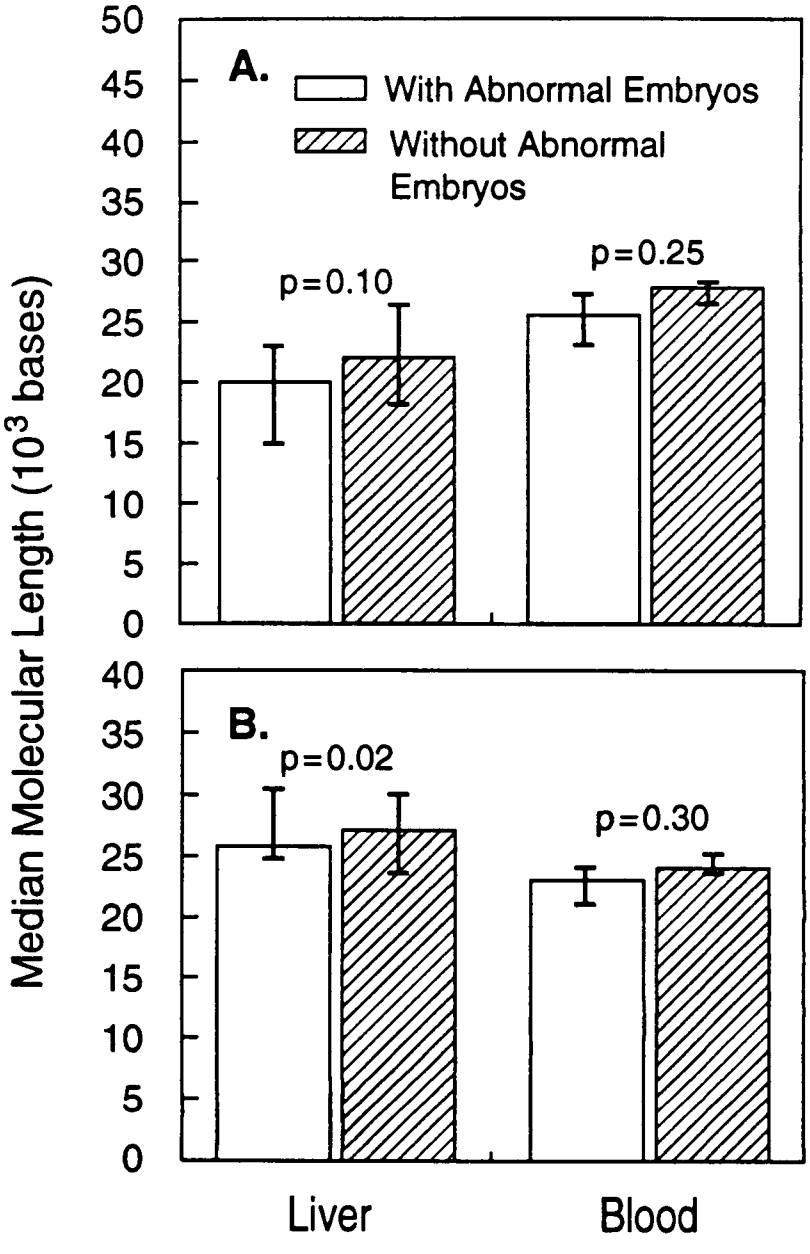
(Pond 3513). A) Total strand breaks in the blood. B) Double strand breaks in the blood. C) Total strand breaks in the liver. D) Double strand breaks in the liver.

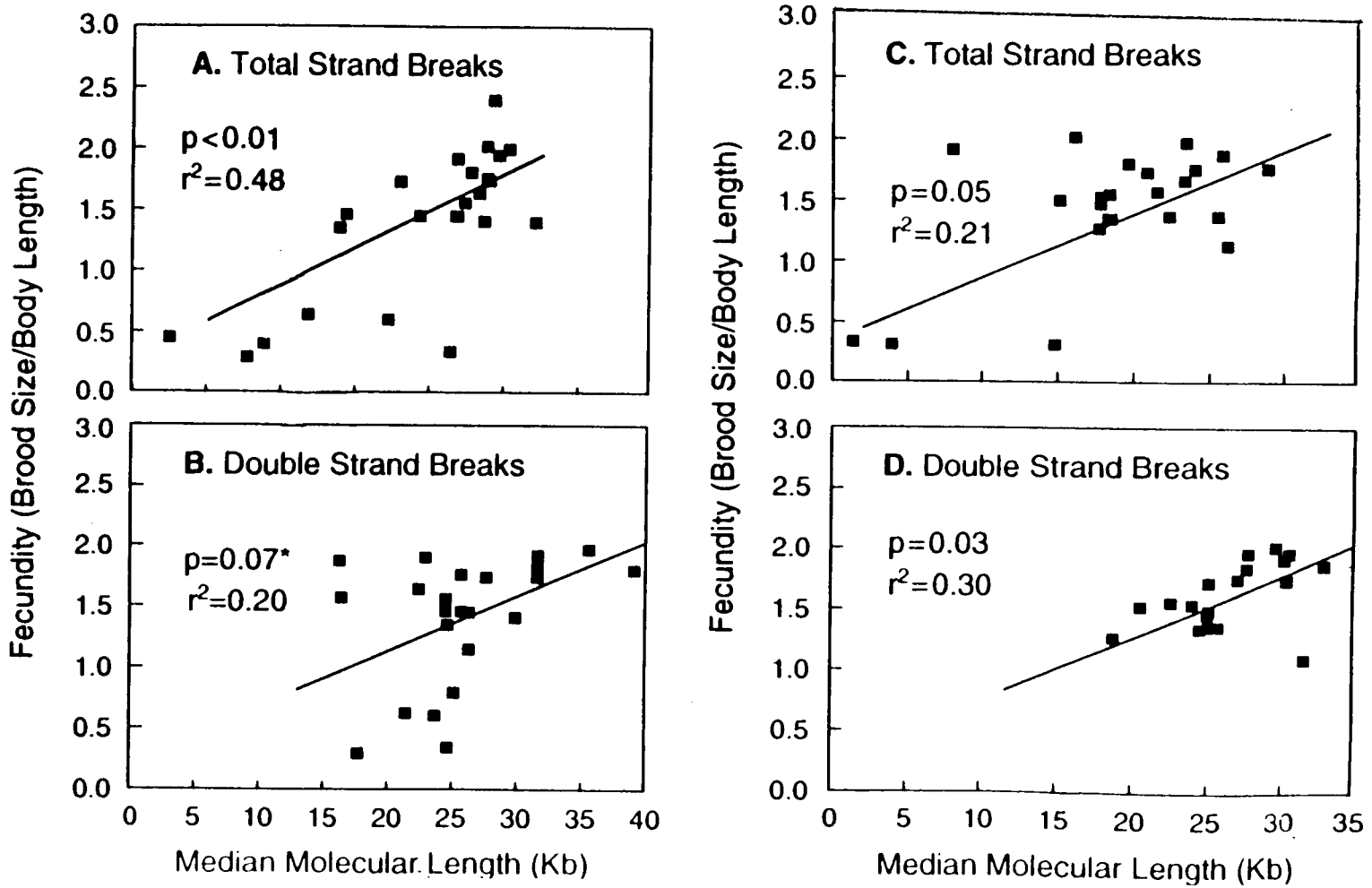
Figure 4 - Median molecular lengths of DNA from mosquitofish collected from Pond 3513 in the spring and summer, 1992. Data are presented for liver and blood cell DNA and for total and double-strand breaks. Values of the bars and error bars represent medians with first and third quartiles. Numbers above each pair of bars denote probability values for the test of $H_o$: no difference between spring and summer values (Wilcoxon rank-sum test).

Figure 5 - Average fecundity of mosquitofish collected from radionuclide contaminated (Pond 3513 and White Oak Lake) and non-contaminated sites in both the spring and summer, 1992. Bars labeled with the same letter are not statistically different within each season (P , 0.05, Kruskall-Wallace test). Bars and error bars represent medians with first and third quartiles.

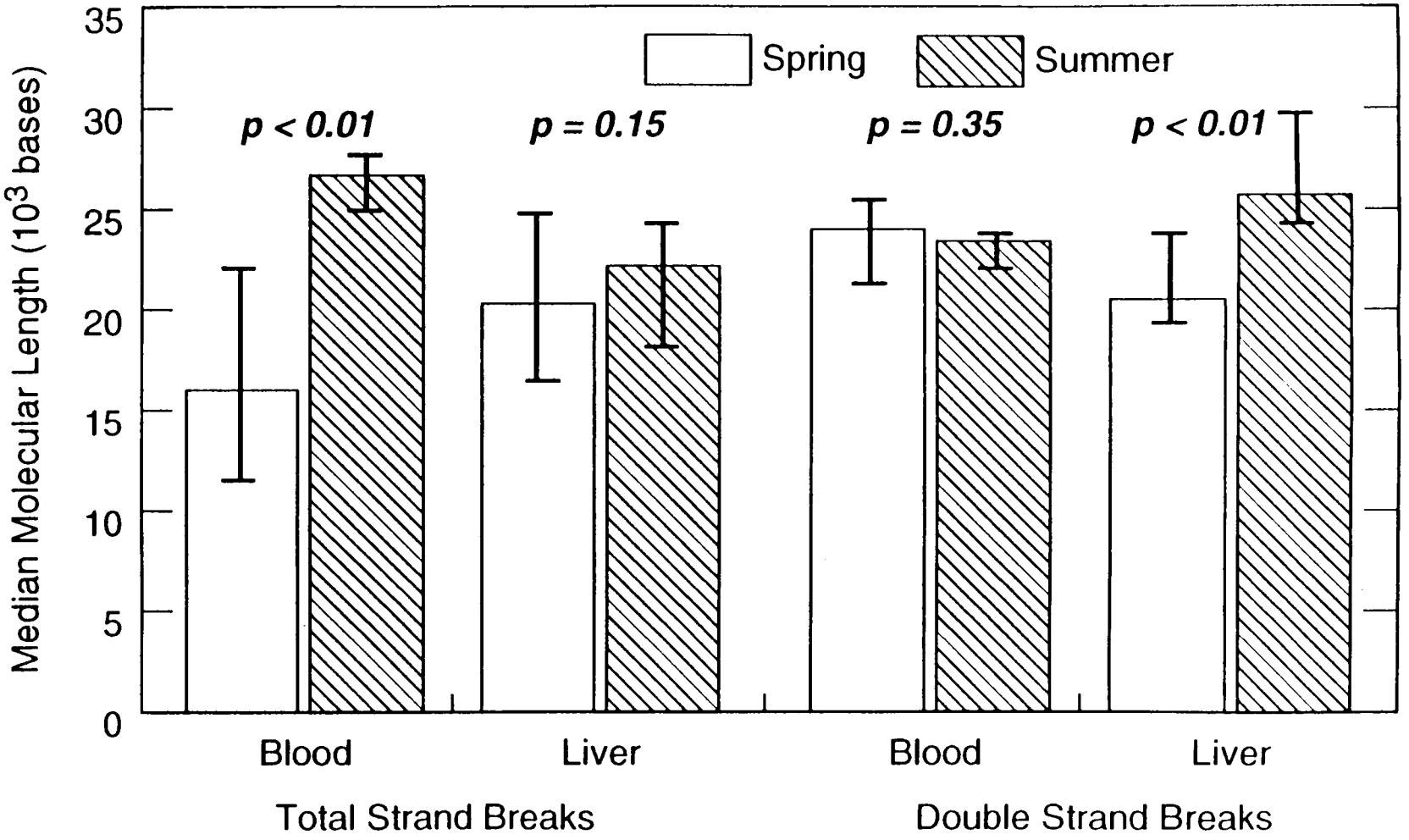

A. Total Strand Breaks
B. Double Strand Breaks
Median Molecular Length ($10^3$ bases)
0
5
10
15
20
25
30
35
40
A
a
B
b
A
c
C
c
White Oak Lake
Pond 3513
Crystal Springs
Wolf Creek
Liver
Blood

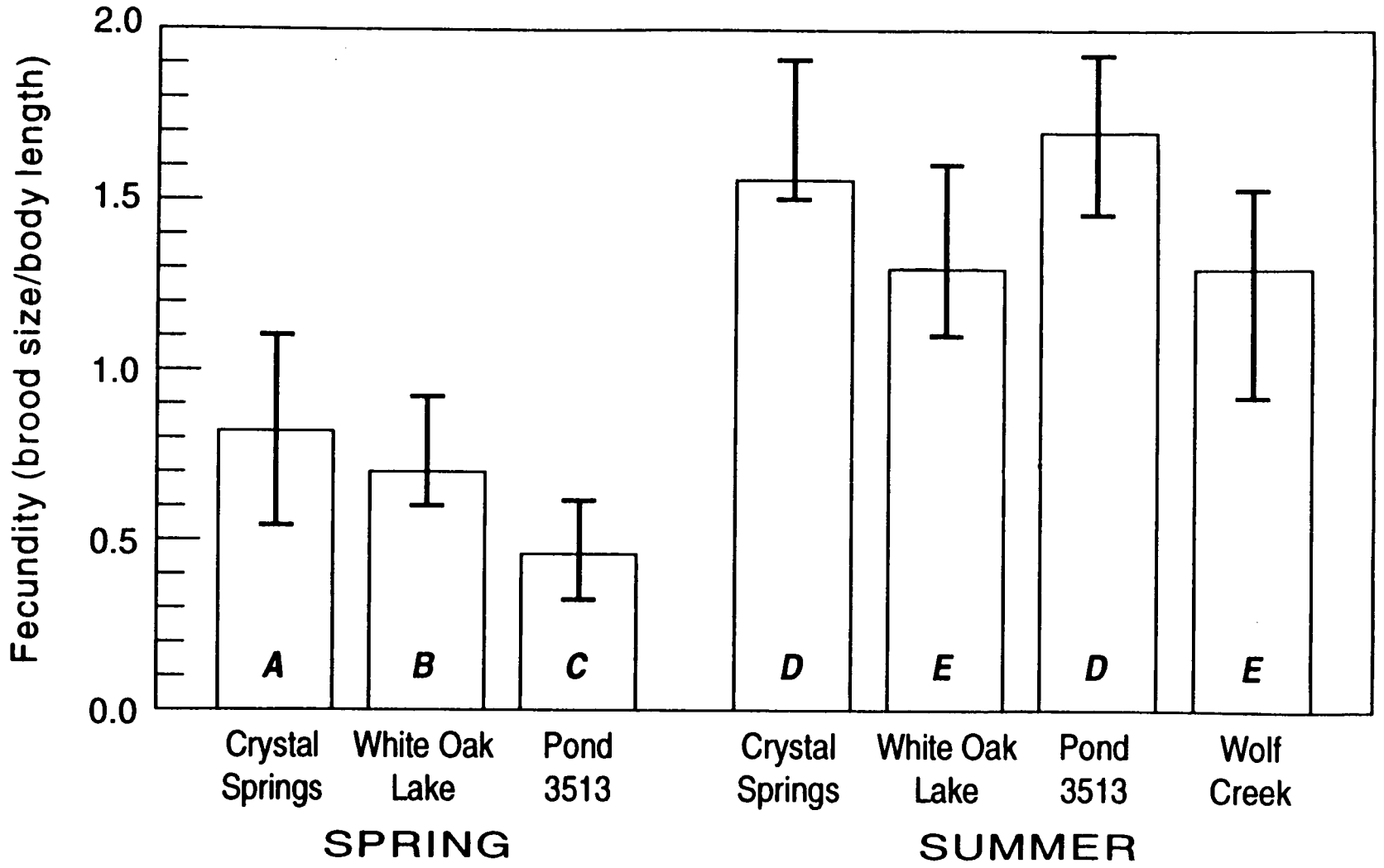
ORNL-DWG 93M-14624R




A. Total Strand Breaks
p<0.01
r²=0.48
B. Double Strand Breaks
p=0.07*
r²=0.20
C. Total Strand Breaks
p=0.05
r²=0.21
D. Double Strand Breaks
p=0.03
r²=0.30
Fecundity (Brood Size/Body Length)
Median Molecular Length (Kb)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20
25
30
35
40

ORNL-DWG 94M-1159




Fecundity (brood size/body length)
2.0
1.5
1.0
0.5
0.0
A
B
C
D
E
D
E
Crystal Springs
White Oak Lake
Pond 3513
Crystal Springs
White Oak Lake
Pond 3513
Wolf Creek
SPRING
SUMMER

# PART 5

# GENETIC ECOTOXICOLOGY OF RADIONUCLIDES IN MOSQUITOFISH II: POPULATION STRUCTURE REVEALED BY RAPD AND ALLOZYME ANALYSES

## JUSTIFICATION

It was suggested in Part 2 that variation in response may lead to selection for resistant individuals in pollution-contaminated environments, and that this may influence population genetic structure. In Part 4 it was found that strand breakage was correlated with fecundity, and that this may provide a venue through which changes in population structure could occur. Further, it was mentioned previously that the results so far have pertained mainly to individual-level response. But in order for biomarkers to be of value in ecological risk assessment, they must be correlated to population-level responses. Therefore this study will examine changes in population genetic structure induced by genotoxicant (in this case radionuclide) contamination.

**ABSTRACT**

In 1977, approximately 250 mosquitofish (Gambusia affinis) were transplanted from a relatively uncontaminated site (Crystal Springs) into a small pond on the Oak Ridge reservation that is heavily contaminated with radionuclides (Pond 3513). DNA polymorphisms, using the RAPD (Randomly Amplified Polymorphic DNA) technique, and allozyme genotypes were examined in order to determine if any genetic differentiation had occurred between the two populations. Also, fish from another radionuclide-contaminated population (White Oak Lake) and two unrelated non-contaminated populations also were examined. The RAPD technique uses the polymerase chain reaction with a short oligonucleotide primer to produce DNA fragments (<2000 nucleotides) of various lengths. When analyzed by gel electrophoresis, these fragments form banding patterns similar to DNA fingerprints. A total of 26 primers were used, 15 of which were found to produce polymorphic banding patterns in at least two populations. These polymorphic primers were used produce a total of 136 bands were produced. Patterns produced by 10 of the polymorphic primers indicated increased diversity in the contaminated sites, while 3 primers indicated decreased diversity. For data generated by all primers, there was an increased diversity in contaminated relative to reference sites. Furthermore, the patterns produced by 6 primers

indicated that there was a higher average number of bands in the contaminated sites than in the non-contaminated sites, and for 3 primers there was a lower average number of bands in contaminated sites. In addition, 17 of the 137 bands were found at a higher frequency in the contaminated compared to the non-contaminated populations. For the allozyme analysis, it was found that there was a higher percent polymorphism and heterozygosity in the contaminated relative to reference sites. These data will be discussed with relation to biomonitoring programs and evolution of resistance to genotoxins in natural populations.

## INTRODUCTION

When one examines the responses of fish or other organisms to toxic stress there is inevitably some variation in response. In some cases the response of one individual may be 2 or 3 times greater in magnitude than the response of another (eg., see part 2). Presumably this is determined in part by genetic variation in terms of protein structure or gene expression. If this is the case then there is a possibility of natural selection in contaminated environments on genetic variants which respond to toxicants to a greater or lesser degree.

There is the possibility that this selection may alter the genetic structure in affected populations. In many cases, allozyme analysis has been employed in order to examine such effects. For example, very low levels of genetic variability in yellow perch in Lake Erie compared to reference lakes have been attributed to selection of pollution-resistant individuals (Strittholt et al. 1988). Reduction in genetic variation supposedly due to environmental contamination has also been found in other aquatic organisms (Kopp et al. 1992, Nevo et al. 1986, Lavie and Nevo 1982). Furthermore, alleles at some loci have been found to increase in frequency in contaminated populations relative to reference sites (Benton and Guttman 1992, Diamond et al. 1989).

The majority of such studies have either addressed the effects on genetic structure from industrial pollution in general (Nevo et al. 1986, Gillespie and Guttman 1989) or specifically heavy metals (Benton and Guttman 1991, Lavie and Nevo 1982, Nevo et al. 1981, Newman et al. 1989), surfactants (Lavie et a. 1982), pesticides (Hughes et al. 1991) or acid stress (Kopp et al. 1982). There has been little, if any, work done on the interactions between genotoxicants and population genetic structure. In this study radionuclides have been chosen as the genotoxin, because such contamination on the Oak Ridge Reservation has been well characterized and the history of such contamination is well known (Blaylock et al. 1991, Tamura et al. 1977). Furthermore, Gillespie and Guttman (1989) have found changes in population genetic structure as the result of radionuclide exposure of stoneroller minnows (Campostoma anomalum). Therefore it is expected that radionuclide contamination would affect the distribution of allozyme genotypes in populations of Gambusia.

Although there have been a number of studies which examine the effects of contaminants on allozyme genotypes, such effects on DNA genotypes have received little attention. In this study, DNA polymorphisms will be examined using the RAPD (Randomly Amplified Polymorphic DNA) technique. This technique uses the polymerase chain reaction with short (10 base pair) oligonucleotide primers to produce DNA fragments. When analyzed by agarose gel electrophoresis, these fragments

produce banding patterns similar to DNA fingerprints or mitochondrial restriction fragment length polymorphisms (RFLPs). These bands have been found to exhibit Mendelian inheritance typically acting as dominant markers (Welsh and McClelland 1990, Williams et al. 1990). Although the RAPD technique has been employed in such endeavors as taxonomy, species identification, parentage assessment (see Hadrys 1992 for review), and endangered species management (Hedrick 1992), its widespread use in population genetic studies has yet to be realized. This is unfortunate, because this technique has several advantages over other currently used techniques. First, it requires much less DNA and does not require the use of radioactive probes as do mitochondrial restriction fragment length polymorphisms (RFLPs) and DNA fingerprinting using genomic RFLPs. Second, it encompasses genetic differences which occur over the entire genome, unlike allozyme studies which only examine a few specific loci. Furthermore, RAPDs are amplified from inverted repeats. Because certain DNA sequences containing inverted repeats have been shown to be responsive to DNA damaging agents (eg. transposons [Doring and Starlinger 1986]), RAPDs may be appropriate for genotoxic study. Finally, RAPDs have been used to produce genetic markers of certain phenotypic characteristics (Michelmore et al. 1991). Thus RAPDs might produce population genetic markers of genotoxicant-contaminated populations.

There are also some drawbacks to the RAPD technique. One is that the genomic identity and possible biological significance of RAPD bands are currently unknown. These bands can be amplified from various locations in the genome, from coding or non-coding regions, or from repetitive or single-copy DNA. Therefore assigning adaptive significance to particular bands should be viewed with caution, at least until rigorous investigation of the genomic locations and possible functions of the amplification sites have been thoroughly characterized. A second drawback is that the banding patterns are sensitive to reaction conditions: changes in reagent composition or PCR thermal cycle profiles may lead to changes in banding patterns. Furthermore, it has been found that in some cases bands are produced which are not reproducible or are difficult to score. Finally, the RAPD bands are usually identified by molecular weight, and not by nucleotide sequence. Therefore two DNA fragments with similar molecular weights but different sequences may be identified as a single band. These drawbacks are further discussed and reviewed in Hadrys et al. (1992) and Williams et al. (1990). However, if the reactions are run in duplicate, the experimenter is careful about cross-contamination of DNA samples, and the reaction conditions are rigidly standardized, the banding patterns can be consistent and reproducible.

Therefore it was felt that the RAPD and allozyme techniques may be useful in examining the relationship between

genotoxicity and population genetic structure. This relationship was examined using the mosquitofish (Gambusia affinis) - a small (< 5 cm), topfeeding, livebearing, freshwater fish. Working with this fish has several advantages. First, it is very fecund, producing at least 3 or 4 broods per year. Thus the population structure of this fish can respond to environmental stressors relatively rapidly. In addition, the ecological genetics of this fish has been well characterized (Lydread et al. 1991, Wooten et al. 1988, McCleneghan et al. 1985, Chesser et al. 1984, Smith et al. 1983). Furthermore, genetic analysis via allozyme polymorphisms has revealed that this species displays genotype-specific responses to xenobiotic stress (Diamond et al. 1989, Newman et al. 1989). Finally, the physiological and demographic responses of Gambusia populations living in contaminated sites on the Oak Ridge Reservation have been well studied (Part 4, Blaylock and Frank 1980, Blaylock and Trabalka 1978).

For this study, four different mosquitofish populations were examined. A population of approximately 250 fish was transplanted in 1977 from a relatively non-contaminated population (Crystal Springs) into a pond heavily polluted with radionuclides (Pond 3513), located on the ORNL grounds. Because Gambusia typically produce 3 or 4 broods per year (Milton and Arthington 1983), there has been at least 48 generations produced in this pond. Such a transplantation

affords a unique opportunity to examine if exposure to radionuclides causes changes in population genetic structure. These populations were compared to unrelated contaminated and uncontaminated populations to assess any changes in the Pond 3513 population since 1977 that could be due to radionuclide contamination.

The objectives of this study are to determine if exposure to radionuclides causes changes in population genetic structure (eg. genetic diversity or genotype frequencies) and if the RAPD and allozyme techniques are appropriate to examine such changes. It is hypothesized that genetic diversity will decrease in genotoxicant-contaminated populations. Furthermore, it is predicted that certain genotypes will have a selective advantage in contaminated populations (in terms of fecundity), and that the frequency of these genotypes will be greater in the contaminated populations.

## METHODS AND MATERIALS

### Chemicals

Taq polymerase was obtained from Promega Corp. (Madison, WI). A 10X assay buffer and 2.5 mM $MgCl_2$ also were supplied with the enzyme. The 10X buffer consisted of 500 mM KCl, 100 mM Tris-HCl, and 1% Triton X-100, pH 7.0. DNA molecular length markers (bacteriophage $\phi$X174 DNA, Hae III digest) were obtained from Promega or Sigma Chemical Corp. (St. Louis, MO). Agarose, Tris-HCl, EDTA, boric acid, phenol, and ethidium bromide were obtained from Gibco-BRL Inc. (Gaithersburg, MD). Deoxynucleotides (dATP, dCTP, dGTP, and dTTP) were obtained from Boehringer Manheim (Indianapolis, IN). Bovine serum albumin (BSA) was supplied by Idaho Technology (Idaho Falls, ID). Chloroform and ethanol were from J.T. Baker (Phillipsburg, NJ). Oligonucleotide primers were obtained from Operon Technologies (Alameda, CA) or from the Nucleic Acid and Protein Service Unit, Biotechnology Laboratory, University of British Columbia (Vancouver, BC). The sources of reagents for allozyme starch gel electrophoresis are reported in Kramer et al. (1992).

### Study Sites

The four populations examined in this study include Pond 3513, White Oak Lake, Crystal Springs and Wolf Creek. Pond 3513 is a 0.45 ha man-made settling basin heavily contaminated with radionuclides such as $^{137}Cs$, $^{90}Sr$, and $^{235}Pu$ (Tamura et al. 1977). White Oak Lake is a 4.7 ha lake created by the damming of White Oak Creek at its confluence with the Clinch River. It is contaminated with radionuclides as well as organic compounds and heavy metals (Blaylock et al. 1991). Crystal Springs is a first-order stream which originates from an underground spring in Oak Ridge, TN. The flow of the stream is impeded by a small concrete weir immediately downstream of its origin, creating a pond approximately 6 ha in area. The stream then continues downstream to Poplar Creek and eventually the Clinch River. Wolf Creek is a first-order stream located near Oak Ridge, TN. At its confluence with the Clinch River, the creek terminates in an approximately 10 ha cattle Pond, which is created by an earthen dam that impedes the flow of water.

**Specimen Collection**

Thirty-five to 40 adult female fish were collected from each site using a seine or dip-net. The fish were transported to the laboratory where blood and carcasses (the head and internal organs removed) were collected and stored at -70° C until analysis. The number of embryos per female also were

counted. Because the brood size of mosquitofish is linearly related to the size of the female (Milton and Arthington 1983), the relative fecundity was determined as brood size/body length. Because adequate fecundity data were not available for all sites, the comparisons between fecundity and genotype will focus on Pond 3513. The DNA from the blood cells was extracted with phenol/chloroform, precipitated in ethanol and redissolved in TE (10 mM Tris, 1 mM EDTA). See Part 4 for a detailed description of specimen collection and DNA extraction procedures. DNA stock solutions were diluted in sterile water to a concentration of 10 ng/ul. All DNA solutions were stored at -20° C until analysis.

**RAPD Analysis**

For RAPD analysis, the reactions were carried out using either a DNA Thermal Cycler 480 (Perkin-Elmer Cetus Corp., Norwalk, CT) or an Air Thermo Cycler 1605 (Idaho Technologies, Idaho Falls, ID). For each particular primer, all populations were examined using the same apparatus. For the Perkin-Elmer apparatus, the reaction mixture consisted of 2.5 mM $MgCl_2$, 100 $\mu$M of each nucleotide triphosphate, 15 ng primer, 10 ng DNA, 1 Unit Taq polymerase and a 1/10 volume of the assay buffer supplied by Promega Corp. (see above) in glass double-distilled water. Reactions were carried out in a volume of 25 $\mu$l. The amplification protocol consisted of 94° C for 1 min,

followed by 40 cycles of 1 min at 94° C, 1 min at 37° C and 2 min at 75° C. This protocol was adapted from Williams et al. (1991). For the Idaho apparatus, the reaction mixture consisted of 2.5 mM $MgCl_2$, 10 mg/ml BSA, 100 $\mu$M of each nucleotide (as above), 12 ng primer, 20 ng DNA and 0.6 units Taq polymerase in glass double-distilled water. The reactions were carried out in a volume of 10 $\mu$l. The amplification protocol consisted of 2 cycles of 1 min at 92° C, 7 sec at 42° C, and 70 sec at 72° C, followed by 38 cycles of 1 sec at 92° C, 7 sec at 42° C, and 70 sec at 72° C. This protocol was adapted from Idaho Technologies (1992). All of the above procedures were carried out using sterile technique. Amplification products were loaded onto a 1.5% agarose gel with 0.5 $\mu$g/ml ethidium bromide and subjected to electrophoresis at 4 V/cm for 1.5 h. The gels were photographed under UV light. An aliquot of molecular length standards (bacteriophage $\phi$X174 DNA digested with Hae III) were run alongside the samples for relative determination of molecular length. When analyzed via gel electrophoresis, a banding pattern reminiscent of RFLPs is produced (Fig. 1). For this analysis, the presence or absence of bands was scored visually.

The data gathered from these photographs included the average number of bands per individual, the frequency of each band (i.e., the proportion of individuals in a population for which each band is produced) and genetic diversity.

Differences between populations in terms of average number of bands were tested using the Kruskall-Wallace test; and in terms of band frequencies using a $\chi^2$ test.

Because the RAPD technique is relatively new and there is no one standard method used routinely for determining genetic diversity, two different methods were used for comparison. One method involved calculation of the band sharing index (BSI, Lynch 1990) using the formula $BSI_{ij} = 2S_{ij} / (N_i + N_j)$, where $S_{ij}$ is the number of bands shared by individuals i and j; $N_i$ and $N_j$ are the number of bands exhibited by individuals i and j, respectively. The BSI was calculated for all i,j pairs and averaged within each population. This index has a value from 0 to 1 when all individuals have completely different or all have the same banding patterns, respectively. Differences between populations were tested by calculating the variance of the mean BSI within each population (Lynch 1990) and using this variance to calculate a standard normal test statistic. Another measure of genetic diversity was the nucleon diversity (h) index developed for use with mitochondrial RFLPs (Nei 1987). This was calculated using the formula:

$$h = [(n /(n - 1)](1 - \sum_{i=1}^{m} x_i^2)$$

where n is the sample size, $x_i$ is the frequency of the $i^{th}$ genotype (banding pattern), and m is the number of genotypes in the population. Differences between populations were

tested by calculating a variance for each population (Nei 1987), and calculating a standard normal test statistic as above.

The nomenclature for each primer and for each band was as follows. The name of each primer consisted of the abbreviation of the company which manufactured the oligonucleotide followed by a number assigned by the manufacturer to identify each primer. Operon technologies produces several sets of 10 base pair oligonucleotides, each set consisting of 20 separate oligonucleotide primers. For this experiment, set D was used, so the primers in this set were identified as OPD. The University of British Columbia primers were identified as UBC. Examples of different primers are OPD1, OPD2, UBC1, UBC2, etc. Each primer has a unique nucleotide sequence. The molecular weight of the bands produced by each primer were calculated using a set of molecular length markers (bacteriophage $\phi$X174 DNA, Hae III digest). The electrophoretic migrational distance and molecular length of each DNA marker fragment was used in an equation to compute the molecular length (in nucleotide bases) of each RAPD fragment (these calculations are reviewed in Weir [1990]). Each band is indicated by the primer and which produced it with its molecular length as its subscript. For example, band $UBC2_{829}$ is a fragment 829 nucleotides long produced by primer UBC2.

Furthermore, selection coefficients were calculated for fish with or without particular bands as follows. First, 17 RAPD bands showed fecundity differences between fish with and without each band in a contaminated population (see results). Therefore, the selection coefficients were calculated using these bands. The relative fitness of fish without these bands (w) was calculated by dividing the average fecundity of fish with these bands by the average fecundity of fish without the bands. For all of the bands for which a selection coefficient was calculated (17 total), the fish in Pond 3513 with the bands had a higher average fecundity than fish without the bands. The selection coefficient = 1- w. Then the correlation between selection coefficients with band frequency for fish from Pond 3513 were calculated and tested with Kendall's test. The rationale for choosing these particular 17 bands is detailed in the next section.

Genetic distances between populations were calculated using the formula for Roger's genetic distance (Nei 1987) using band frequencies instead of allele frequencies. The distance between populations i and j was calculated with the following formula (Nei 1987):

$$D_{ij} = (1/n) \sum_{k=1}^{n} (x_{ik} - x_{jk})^2$$

where n = the number of bands, $x_{ik}$ is the frequency of the $k^{th}$ band in the $i^{th}$ population, etc. This index has a value of 0

to 1 when the populations are completely identical and completely different, respectively.

Distances were calculated for each pair of populations. Statistical tests for discriminating between different pair-wise comparisons can be problematic (Nei 1987). Therefore, the distances for each locus were treated as a sample, and the differences between different pair-wise comparisons were then tested using a Student's t-test, as suggested by Nei (1987).

**Allozyme Analysis**

The methods for allozyme analysis were as described in Diamond et al. (1989). Briefly, the whole carcass of each fish was homogenized with a glass rod in approximately 50-150 $\mu$l of buffer (10 mM Tris, 1 mM EDTA, 50 mM NADP, pH 7.0) and centrifuged at 12000 rpm in a microcentrifuge for 45 sec. Paper wicks were then saturated with supernatant, loaded into a 12.5% starch gel and subjected to electrophoresis for at least 10 h. The gels were then stained with the appropriate reagents to visualize each enzyme system. Conditions for electrophoresis and staining of each enzyme can be found in Harris and Hopkinson (1976), Ayala et al. (1972) and Selander et al. (1971). Allelic variants of each enzyme were scored as fast (F) or slow (S), depending on their relative mobility. Homozygotes were given the connotation FF or SS, while heterozygotes where depicted as FS.

Genetic variation was measured as degree of heterozygosity or percent polymorphism. Differences in heterozygosity were tested by calculating the variance for the heterozygosity estimate (Weir 1990) and using this variance to calculate a standard normal test statistic. Differences in percent polymorphism between populations were tested using a $\chi^2$ test. Genetic distances (D) between populations were calculated using Roger's distance (Nei 1987). The differences between distances between each pair of populations were tested by treating each locus as a sample and preforming a t-test (Nei 1987).

## RESULTS

For the results below, and comparisons or correlations involving fecundity concentrated on Pond 3513, because adequate fecundity data was not available for the other populations.

### RAPD Analysis

Forty different primers were initially used to screen for polymorphic banding patterns. These primers included OPD1 to OPD20 and UBC1 to UBC20. Of these, 24 were found to produce banding patterns which were polymorphic in at least one population. Of these, 9 primers produced patterns which were polymorphic in only one population, and in these populations there was only a very small percentage (<5%) of polymorphic individuals. Therefore these primers were eliminated from the analysis. The results using the remaining 15 primers are reported here.

Genetic Diversity - The genetic diversity as revealed by the RAPD assay showed higher diversity within the contaminated sites relative to reference sites for 10 of 15 the primers examined (Table 1[4]). For 3 of the primers (OPD8, OPD12,

[4]All tables and figures to Part 5 are found in the appendix to Part 5.

OPD13), there was a lower diversity in the contaminated sites, and there were no trends with regard to contaminated vs. reference sites for 2 of the primers (UBC1 and UBC2; Table 1). Averaged over all primers, the diversity is greater for contaminated than for reference sites (Table 1).

Number of Bands - The average number of bands was greater in contaminated populations than for non-contaminated populations for 6 primers (OPD2, OPD7, OPD20, UBC4, UBC9, and UBC12; Table 2), the average number of bands was less in contaminated populations for 3 primers (OPD8, OPD12 and OPD13), and for the remaining 6 there were no difference between populations.

For 3 of the 6 primers which indicated that there was a higher number of bands in contaminated populations, there was a positive correlation between allozyme heterozygosity and RAPD bands using individuals from the Pond 3513 population (Fig. 2): individuals which where heterozygous for at least one locus had a higher number of bands than did completely homozygous individuals. In addition, this same relationship was true for 3 of the 9 remaining primers (Fig. 2).

In the Pond 3513 population, there was a positive correlation between fecundity and number of bands (i.e., individuals with more bands had a higher fecundity) for 4 primers at $P \leq 0.05$ and 2 at $0.05 < P \leq 0.10$ (Table 3). There

were no statistically significant correlations between number of bands and fecundity for any of the other primers.

Band Frequency - In this analysis, there were a total of 139 bands produced by the 15 primers. The molecular lengths of each of these bands is indicated in Table 4. Four of these 139 bands decreased in frequency in both contaminated sites relative to reference sites (Table 5). Seventeen of these bands increased in frequency in both of the contaminated populations relative to both of the non-contaminated populations (Table 5). There also were fecundity differences between fish with and without 8 of these 17 bands: fish from Pond 3513 which possessed at any one of these 8 bands had higher fecundity than fish which did not (Table 6).

For the 17 bands with increased frequency in contaminated sites, there was a positive correlation between their frequency and selection coefficients in Pond 3513 (Fig. 3).

Genetic Distance - When genetic distances were calculated for each individual primer, there was no overall pattern common to all primers. Three primers showed the greatest similarity between White Oak Lake and Pond 3513, 3 showed the greatest similarity between Pond 3513 and Crystal Springs, 2 primers showed the greatest similarity between Crystal Springs and Wolf Creek, and 1 primer showed the greatest similarity between White Oak Lake and Crystal Springs (Table 7). For the

remaining primers and distances, there was no single distance that was significantly different from all others.

When all primers were averaged together, it was found that there were no differences in the distances between Pond 3513, White Oak Lake, and Crystal Springs (Table 8), but they were all different from Wolf Creek. Furthermore, all of the average distances between contaminated and control sites were pooled in order to get an overall distance between contaminated and control sites. This overall distance also was compared to the average distances between the two contaminated sites and between the two control sites. The distance between the two contaminated sites (0.29) was lower than the overall distance between control and contaminated (0.32), which was in turn lower than the distance between the two control sites (0.35; $P \leq 0.05$, Student's t-test).

**Allozyme Analysis**

Genetic Diversity - In general, the levels of heterozygosity were very low for most loci. There were twelve polymorphic loci found: malate dehydrogenase (MDH, three loci: MDH1, MDH2, MDH3); fumarase (FUM); isocitrate dehydrogenase (IDH); nucleoside phosphorylase (NP); hydroxybutarate dehydrogenase (HBDH); lactate dehydrogenase (LDH); adenosine deaminase (ADA); phosphoglucomutase (PGM); glucose - 6 - phosphate dehydrogenase (GPD); and lysyl-alanine peptidase

(PEP). The heterozygosity at the nucleoside phosphorylase (NP) locus was an order of magnitude greater than that of the other loci, so the results for this locus were analyzed separately from all other loci. Besides the NP locus, the only polymorphic loci in Pond 3513 were FUM, HBDH, and GPD, with percent heterozygosities of 0.085, 0.067, and .067, respectively. In Crystal Springs, NP was the only polymorphic locus. In White Oak Lake, the polymorphic loci besides NP were MDH1, MDH2, HBDH, LDH, and ADA with heterozygosities of 0.082, 0.040, 0.040, 0.082, 0.061, and 0.041, respectively. In Wolf Creek, the only polymorphic loci besides NP were MDH1 and LDH, with heterozygosities of 0.076 and 0.051, respectively. Genotypic distributions of all loci were in agreement with Hardy-Weinberg expectations.

Both the levels of heterozygosity and percent polymorphism were higher in the contaminated sites than in non-contaminated sites (Fig. 4). Within the Pond 3513 site, heterozygous individuals had a higher fecundity than did homozygous individuals (Fig. 5), both for NP ($P=0.02$) and for all other loci ($P<0.01$, Wilcoxon rank-sum test). In addition, for the NP locus the frequency of the FS genotype was higher and the frequency of the SS genotype was lower in the contaminated than in the non-contaminated populations (Fig. 6).

Genetic Distance - When genetic distances were calculated using all allozyme allele frequencies, there was no difference in the distances between White Oak Lake, Pond 3513, and Crystal Springs (Table 9). The greatest similarity was between Pond 3513 and Crystal Springs, or Pond 3513 and Wolf Creek. The least similarity was between Wolf Creek and Crystal Springs. When distances were calculated using only the nucleoside phosphorylase locus, the smallest distance was between White Oak lake and Pond 3513 (Table 9). For all loci and for the nucleoside phosphorylase locus, the data for the two contaminated sites were pooled, as were the data for the two non-contaminated sites, in order to obtain an overall distance between contaminated and reference sites. For all loci, this overall distance (0.021) was lower than the distance between the two contaminated sites (0.036), which was in turn lower than the distance between the two non-contaminated sites (0.054; $P \leq 0.05$, t-test). For the NP locus, the distance between the two contaminated sites (0.035) was less than the distance between the two reference sites (0.047), which was in turn less than the overall distance between control and contaminated sites (0.076; $P \leq 0.05$, t-test).

## DISCUSSION

The results of this study provide evidence that population genetic structure is affected by exposure to radionuclides, supporting the original hypothesis. In particular, the use of RAPDs appears to be able to reveal differences between contaminated and non-contaminated populations. The differences between Pond 3513 and Crystal springs in terms of genetic diversity, number of RAPD bands, frequency of RAPD bands, heterozygosity and percent polymorphism suggested that changes in population genetic structure have occurred since Pond 3513 was colonized from Crystal Springs in 1977. For these parameters, RAPD and allozyme patterns found in Pond 3513 are more similar to White Oak Lake than they are to Crystal Springs, suggesting the possibility that these changes may be influenced by radionuclide contamination. Such similarities between Pond 3513 and White Oak Lake were found for genetic diversity (11 primers showed this trend), number of bands (9 primers) and band frequencies (17 of the bands studied showed this pattern). Similarities also were found for the nucleoside phosphorylase allozyme locus. In such cases, the patterns of genotypes in Crystal Springs were more similar to those of Wolf Creek population, which was also non-contaminated. This provides evidence in support of the hypothesis that these changes may be due to radionuclide-induced selection. Because

this is a preliminary study and the number of populations studied was small, differences between Pond 3513 and Crystal Springs due to random drift or other aspects of neutral evolution cannot be ruled out, and more populations need to be examined to further verify these results.

**RAPD Analysis**

For the RAPD assay, the three principal patterns found were changes in genetic diversity, changes in average number of bands per individual, and differences in the frequency of certain bands between contaminated and noncontaminated populations. In most cases the direction of the patterns were highly primer specific, but some generalities can be made.

Genetic Diversity - Genetic diversity as revealed by the RAPD and allozyme techniques was higher in the contaminated populations. This is the reverse of what was hypothesized and what was found in other studies (Kopp et al. 1992, Strittholt et al. 1988, Nevo et al. 1986). However, this finding is supported by other studies. An apparent increase in RAPD diversity has also been seen in sunfish populations exposed to paper mill effluents (Kai-Lin Lee, Oak Ridge National Laboratory, unpublished data). Furthermore, de novo formation of increased genetic variation has been found for several different species experiencing environmental stress (reviewed

in Würgler and Kramers 1992). An explanation for the increase in genetic diversity in contaminated populations may be due to genomic rearrangement. For example, it has been found that exposure to X-rays induces transpositions in corn (Zea maize, Doring and Starlinger 1986). Also, such rearrangements as deletions, insertions and transpositions in are known to be induced in fish by ionizing radiation (NCRP 1991, Blaylock and Trabalka 1978, Purdom and Woodhead 1973).

Another possibility is that this pattern may be due to loss of some amplification sites due to mutations. Because the sites of mutations depend on random chance, at least to some extent, not all mutations would take place in the same priming sites. Thus different priming sites may be lost in different individuals, leading to increased genetic variability.

Conversely, 3 primers produced banding patterns with lower diversity in contaminated than in non-contaminated sites, in accordance with other studies (Kopp et al. 1992, Strittholt et al. 1988, Nevo et al. 1986) and with the original hypothesis stated earlier. Whether or not a particular primer shows increased or decreased diversity may depend upon the function and location of the inverted repeats that form the amplification sites. The important point is that both contaminated sites show similar patterns of genetic diversity, whether it is greater of less than that in the reference sites.

Number of Bands - The most common pattern is that fish from the contaminated populations have a higher number of bands per individual than do the non-contaminated populations. This occurred for 6 of the 15 primers examined. Also, fecundity of individuals was positively correlated with the number of bands in the Pond 3513 site for 4 of the primers. This is consistent with the hypothesis that differences between contaminated and non-contaminated sites are due to radionuclide-induced selection.

The reasons for the differences in RAPD patterns in contaminated and non-contaminated sites are not known at this point, but there are several possibilities. One possibility is that differences in genotypes are somehow related to resistance to or repair of the effects of ionizing radiation. For example, it has been found that the amount of DNA strand breakage in some cases is negatively correlated with the number of bands (see part 4 of this dissertation). Perhaps certain genotypes are more adept at repairing or resisting DNA damage than others. This may be due to the fact that RAPDs are amplified from inverted repeats, and inverted repeats may be involved in genotoxic response (see Part 6 for a discussion).

A different alternative is that an increase in the number of bands is somehow related to an increase in heterozygosity. It was found in this study that fish in contaminated habitats had higher allozyme heterozygosity. Furthermore, fish with at

least one heterozygous locus had a higher average number of bands than did homozygous individuals. This was true for 4 of the 6 primers that showed an increased number of bands in the two contaminated sites, as well as for 3 others.

Although an increase in the number of bands was the most common pattern in contaminated populations, for three (OPD8, OPD12, OPD13) primers there was a lower average number of bands per individual in contaminated relative to uncontaminated reference sites. A decrease in the number of bands in contaminated sites may be due to mutation or other DNA lesions in the amplification sites. For instance, by using a technique (AP [Arbitrarily Primed] - PCR with tRNA gene primers) which is very similar to RAPDs, Kubota et al. (1992) found that abnormal embryos of irradiated fish lost some of the parental bands. They suggested that this was due to mutations or other DNA lesions. Thus, it could be that a decrease in the number of bands in the present study may be due to a similar phenomenon. The decrease in these bands in contaminated populations is probably not attributable to selective advantage of individuals with fewer bands. If this were the case, then the correlation coefficient between number of bands and fecundity would be negative (i.e., fish with fewer bands would have a higher fecundity). However, for primers OPD8 and OPD13 the correlation coefficient was positive, although significant at the 0.10 but not the 0.05 level.

Band Frequencies - A third pattern reveled by the RAPD technique is that there were 17 bands that have a higher frequency in contaminated than non-contaminated populations. In these cases the differences between Pond 3513 and Crystal Springs were mirrored by differences between White Oak Lake and Wolf Creek. This provides evidence in support of the hypothesis that the changes in the Pond 3513 population which have occurred since its transplantation from Crystal Springs are due at least in part to radionuclide contamination. One possible explanation for selection of individuals with certain bands is that there may be some RAPD sites which are linked to genes that confer an evolutionary advantage in polluted environments. RAPD amplification sites which are linked to functional genes have been found in other organisms (eg. Michelmore et al. 1991). If the mosquitofish bands are amplified from sites in or near genes which confer a selective advantage in contaminated environments, one would expect that the frequency of such bands would be correlated to the selective coefficient. This is what was found for fish living in Pond 3513. At this point in time, however, caution should be used in assigning a particular adaptive value to specific RAPD genotypes, because not enough is known about them.

Genetic Distance - Besides differences in genotype and diversity between contaminated and non-contaminated populations, there were some specific patterns in terms of

genetic distance for the four populations. For the genetic distances via the RAPD technique, there are primer-specific patterns but some generalities do emerge. For 3 primers the Pond 3513 is the most closely "related" to White Oak Lake. Since both sites are contaminated with radionuclides, this supports the hypothesis of contaminant-induced selection. For 3 other primers, the distance between Crystal Springs and Pond 3513 is the smallest distance. This is not surprising, since Pond 3513 was colonized from Crystal Springs and they are related. The determining factor for whether or not the banding patterns of Pond 3513 population are more similar to Crystal Springs or White Oak Lake may be whether or not certain primer-specific genotypes have a selective advantage over others in the population. In one case the smallest distance was between Crystal Springs and White Oak Lake. The explanation behind this pattern is currently unknown, but possibilities include gene flow, common origins, environmental similarities (eg. Crystal Springs and White Oak Lake are similar for the amount of total DNA strand breakage in the liver, see Part 4). When all primers were averaged together, though, there were no differences for the distances between Crystal Springs, White Oak Lake and Pond 3513. Thus the small distances between White Oak Lake and Pond 3513 may be due to the fact that the distance between White Oak Lake and Crystal Springs was low before the transplantation to Pond 3513.

When the overall genetic distance between contaminated and reference sites were compared to the distance between both contaminated or both reference sites, the order of increasing distance was: both contaminated < overall (contam. vs. ref.) < both reference. This is consistent with the hypothesis that the contaminated sites should be more similar to each other than to the reference sites.

**Allozyme Analysis**

Genetic Diversity - An interesting aspect of this study is that both allozyme and RAPD analyses indicated that there was a higher level of genetic variability in the contaminated populations. As mentioned before, this is the converse of the original hypothesis. One possible explanation may be an increased mutation rate. Alternatively, an increase in heterozygosity may somehow be related to an increase in chromosomal rearrangements. For example, Quitana and Prevosti (1991) found that Drosophila with inversion heterozygotes had an increased number of heterozygous allozyme loci. Blaylock (1965, 1966) found that there was an increased number of unique inversion heterozygotes in Chironomid larvae in White Oak Lake relative to reference sites, so perhaps the aforementioned relationship exists for mosquitofish in White Oak Lake and Pond 3513. Additionally, it has been found in laboratory studies that fish which exhibit higher levels of

heterozygosity are more resistant to xenobiotic stress (Kopp et al. 1992). The same could be true for genotoxic stress, leading to a selective advantage for heterozygotes in contaminated environments. This hypothesis is supported by the fact that heterozygotes have higher fecundity than do homozygotes within Pond 3513.

As mentioned before, the present study has found that heterozygosity and percent polymorphism was increased in contaminated populations, while previous studies have found otherwise. This could be due to differences in the type of contamination examined: perhaps the mutation rate in radionuclide-contaminated populations is greater than that for other contaminants. It could also be due to the fact that the heterozygosity of the fish in this study was roughly an order of magnitude less than that found in other studies (Kopp et al. 1992, Stritholt et al. 1988, Nevo et al. 1986). It could be that the response of populations to environmental contaminants according to environmental conditions, type and amount of contaminant, life history traits of the species examined, or background levels of heterozygosity.

As of yet, though, it has not been determined that differences in diversity between contaminated and non-contaminated populations are due solely or even primarily to radionuclide contamination. Other processes may very well be involved in producing differences in diversity between the populations. For example, there could be a relationship

between genetic polymorphism and habitat heterogeneity (De Meeus et al. 1993) or between genetic variability and population density (Nei 1987). Alternatively, the Pond 3513 population could have undergone a bottleneck upon introduction from Crystal Springs. It has been argued in the past that bottlenecks should decease variability (Fisher 1930), but some recent evidence suggests the opposite, both for single-locus (Leberg 1992) and quantitative traits (Lopez-Fanjul and Villaverde 1989, Bryant et al. 1986). It is clear that more work needs to be done to determine if differences in heterozygosity between sites are due to neutral or selective mechanisms.

Most of this difference in heterozygosity between contaminated and non-contaminated sites are due to the nucleoside phosphorylase (NP) locus. It appears that selection may be acting to increase heterozygosity at the NP locus, because NP heterozygotes in Pond 3513 have a higher fecundity than do homozygotes. A possible link between NP and genotoxicity is that nucleoside phosphorylase is involved in the synthesis of nucleotides, a requisite for DNA repair. It could also be that the NP locus showed the most difference between sites because it had the highest heterozygosity. In any case, the relationship between DNA damage and NP or other enzymes involved in nucleic acid metabolism deserves further attention.

Genetic Distance - The fact that there was no difference between the Pond 3513-Crystal Springs, Pond 3513-White Oak Lake, or White Oak Lake-Crystal Springs distances is in accordance with the findings from the RAPD assay. The finding that the smallest distance using nucleoside phosphorylase only was between Pond 3513 and White Oak Lake is in accordance with the hypothesis that this locus is responsive to selection by genotoxic agents.

When the overall distance between contaminated and reference sites were compared to the distances between both contaminated or both reference sites the order of increasing distance over all loci was: overall (contam. vs. ref.) < both contaminated < both reference. This is not consistent with the hypothesis that the contaminated sites are more similar to each other than they are to the reference sites. However, for the NP locus the order of increasing distance was: both contaminated < both reference < overall (contam. vs. ref.). This is consistent with the hypothesis mentioned above.

**Conclusions**

Obviously the results of the RAPD assay can be highly primer specific, but the important point is that in the above examples both contaminated sites show similar trends, and both the non-contaminated sites show similar trends. But because these patterns can be primer-specific, the trends for each

primer must be characterized using known contaminated and non-contaminated sites before this assay has any utility for biomonitoring.

The results presented here provide evidence that radionuclide contamination may influence population genetic structure. Also, RAPD or allozyme genetic markers may be useful in identifying populations which are undergoing selection from radionuclide-induced stress, perhaps by examining the number of RAPD bands, frequency of certain bands, nucleoside phosphorylase heterozygosity, overall heterozygosity or percent polymorphism in different populations. However, widespread use of RAPD markers for biomonitoring must be viewed cautiously at this point, because we do not as of yet know the genetic location or the possible functions of the amplification sites from which the RAPD bands are produced. Also, the ecological ramifications of altered RAPD patterns is unknown. This makes interpretation of the results obtained from this assay difficult.

**Future Directions**

The results presented in this study, although compelling, are only preliminary. As mentioned above, more work needs to be done before RAPDs can confidently be used in routine environmental monitoring work. It should be noted that the possibility that differences in RAPD patterns due to neutral

variation, random drift or influences from other environmental variables has not been ruled out. More investigation needs to be done along these lines as well. One aspect which was missing from this study were long-term laboratory exposures or caging studies to try to control for environmental or genetic variables which could affect the response. These type of experiments would be most useful in comparing fecundity or some other aspect of fitness between genotypes. Other avenues of future research include: 1) Further verification of the effects of xenobiotic exposure on population RAPD patterns using additional populations, primers and species. 2) Examination of the ecological consequences of changes in RAPD patterns or other measures of population genetic parameters in contaminated populations. 3) Further examination the inter-relationships between fecundity, RAPD patterns, and allozyme patterns in contaminated environments. 4) Investigation of the identity, genomic location, possible function and inheritance characteristics of RAPD banding patterns using laboratory exposures, molecular biology and cytogenetic techniques, and breeding studies. 5) Comparison of results from the RAPD assay with those from other genetic assays such as mitochondrial and genomic DNA restriction fragment length polymorphisms in order to confirm and elucidate RAPD results. In particular, mitochondrial analysis should be interesting because animal mitochondrial DNA does not contain sites for genomic rearrangement. Thus comparisons with mitochondrial

studies may give insights into whether changes in genetic diversity using the RAPD assay are actually due to patterns of genetic relatedness or to patterns of genomic rearrangements.

6) Investigation into the effects of variables such as environmental variability, habitat heterogeneity, population structure and population dynamics on RAPD banding patterns and variability.

## ACKNOWLEDGEMENTS

I would sincerely like to thank B.G. Blaylock, K.L. Lee and R. Clark for their assistance with field collections. This project was sponsored in part by the Oak Ridge Y-12 Plant, Department of Environmental Management, Health, Safety, Environment and Accountability Division and by an appointment to the U.S. Department of Energy Laboratory Cooperative Postgraduate Research Training Program administered by the Oak ridge Associated Universities. This project was also supported in part by the U.S. Army Biomedical Research and Development Laboratory (DOE project No. 1061-BO47-A1). The Oak Ridge National Laboratory is administered by Martin Marietta Energy Systems Inc. under contract DE-AC05-A4OR21400 with the U.S. Department of Energy. Portions of this study were presented at the 13$^{th}$ annual conference for the Society of Environmental Toxicology and Chemistry, Nov 1993.

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# APPENDIX

Table 1 - Two measures of genetic diversity, band sharing indices (BSI) and nucleon diversity index (h) for two radionuclide-contaminated and two non-contaminated mosquitofish populations using the RAPD technique. Pond 3513 and White Oak Lake are contaminated with radionuclides. For each primer and index, populations with similar alphabetical superscripts are not significantly different ($P<0.05$, Kruskall-Wallace test) sample sizes for each population are as follows: Pond 3513, 40; White Oak Lake, 40; Crystal Springs, 38; Wolf Creek, 35. Values with similar alphabetical superscripts are not significantly different ($P<0.05$, Kruskall-Wallace test).

| | | Population | | | |
|---|---|---|---|---|---|
| Primer | Index | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
| OPD2** | BSI* | $0.033^{a}$ | $0.013^{b}$ | $0.135^{c}$ | $0.065^{d}$ |
| | h | $0.454^{a}$ | $0.060^{b}$ | $0.813^{c}$ | $0.662^{d}$ |
| OPD7§ | BSI | $0.077^{a}$ | $0.446^{b}$ | $0.120^{c}$ | $0.027^{d}$ |
| | h | $0.718^{a}$ | $0.969^{b}$ | $0.409^{c}$ | $0.256^{d}$ |
| OPD8§ | BSI | $0.280^{a}$ | $0.290^{a}$ | $0.140^{b}$ | $0.070^{c}$ |
| | h | $0.942^{a}$ | $0.915^{b}$ | $0.847^{c}$ | $0.616^{d}$ |
| OPD11§ | BSI | $0.230^{a}$ | $0.360^{b}$ | $0.130^{c}$ | $0.040^{d}$ |
| | h | $0.933^{a}$ | $0.901^{b}$ | $0.624^{c}$ | $0.191^{d}$ |
| OPD12§ | BSI | $0.420^{a}$ | $0.300^{b}$ | $0.350^{c}$ | $0.300^{d}$ |
| | h | $0.339^{a}$ | $0.517^{b}$ | $0.887^{c}$ | $0.714^{d}$ |
| OPD13§ | BSI | $0.170^{a}$ | $0.320^{b}$ | $0.090^{c}$ | $0.080^{c}$ |
| | h | $0.769^{a}$ | $0.855^{b}$ | $0.801^{c}$ | $0.229^{c}$ |
| OPD18§ | BSI | $0.120^{a}$ | $0.360^{b}$ | $0.110^{a}$ | $0.210^{c}$ |
| | h | $0.894^{a}$ | $0.897^{a}$ | $0.766^{b}$ | $0.621^{c}$ |

Table 1 (cont')

| Primer | Index | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|---|---|
| OPD20§ | BSI | 0.200[a] | 0.230[a] | 0.100[b] | 0.040[c] |
| | h | 0.884[a] | 0.628[b] | 0.421[c] | 0.247[c] |
| UBC1§ | BSI | 0.200[a] | 0.350[b] | 0.120[c] | 0.490[d] |
| | h | 0.901[a] | 0.913[a] | 0.884[b] | 0.829[c] |
| UBC2 | BSI | 0.126[a] | 0.317[b] | 0.190[c] | 0.197[c] |
| | h | 0.763[a] | 0.576[b] | 0.570[b] | 0.883[c] |
| UBC4** | BSI | 0.090[a] | 0.280[b] | 0.420[c] | 0.370[c] |
| | h | 0.789[a] | 0.942[b] | 0.951[b] | 0.960[c] |
| UBC9§ | BSI | 0.160[a] | 0.300[b] | 0.180[a] | 0.250[c] |
| | h | 0.909[a] | 0.901[a] | 0.653[b] | 0.849[c] |
| UBC12§ | BSI | 0.420[a] | 0.300[b] | 0.350[b] | 0.300[b] |
| | h | 0.963[a] | 0.989[b] | 0.872[c] | 0.760[d] |
| UBC16§ | BSI | 0.210[a] | 0.370[b] | 0.130[c] | 0.200[a] |
| | h | 0.943[a] | 0.911[a] | 0.927[a] | 0.893[b] |
| Average† | BSI | 0.247 | 0.317 | 0.213 | 0.177 |
| | h | 0.808 | 0.800 | .751 | .633 |

*Because the BSI is inversely proportional to diversity, the values presented here are 1 - BSI.

**For these primers both contaminated sites had lower diversity than either non-contaminated site as determined by BSI and/or h.

Table 1 (cont')

§For these primers both contaminated sites had higher diversity than either non-contaminated site as determined by BSI and/or h.

†Averaged over all primers

Table 2 - Median (first quartile, third quartile) number of bands produced by the RAPD technique with various oligonucleotide primers. The data include two radionuclide-contaminated (Pond 3513 and White Oak Lake) and two non-contaminated (Crystal Springs and Wolf Creek) populations. For each primer, populations with similar alphabetical superscripts are not significantly different ($P > .05$, Kruskall Wallace test). The sample sizes for each population are as follows: Pond 3513, 40; White Oak Lake, 40; Crystal Springs, 38; Wolf Creek, 35.

| Primer | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|---|
| OPD2* | 8 (8,9)[a] | 8 (8,11)[a] | 5 (4,6)[b] | 6 (5,7)[b] |
| OPD7* | 7 (6,8)[a] | 6 (4,8)[b] | 4 (0,5)[c] | 5 (5,6)[d] |
| OPD8** | 6 (5,8)[a] | 6 (5,7)[a] | 7 (6,8)[b] | 7 (6,8)[b] |
| OPD11 | 5 (4,6)[a] | 8 (6,9)[b] | 8 (6.5,8)[b] | 4(3,4)[a] |
| OPD12** | 1 (0,3)[a] | 1 (0,1)[b] | 3 (2,5)[c] | 3 (2,4)[c] |
| OPD13** | 6 (5,7)[a] | 5 (1,6)[a] | 7 (6,8)[b] | 7 (6,7)[b] |
| OPD18 | 8 (7,9)[a] | 6 (4,7)[b] | 7 (6,9)[a] | 6 (3,6)[b] |
| OPD20* | 6 (5,7)[a] | 6 (5,7)[a] | 4 (4,5)[b] | 5 (5,6)[b] |
| UBC1 | 7.5 (6,8)[a] | 5 (3,6)[b] | 8 (7,9)[a] | 6 (2,7)[b] |

Table 2 (cont')

| Primer | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|---|
| UBC2 | 5 (4,6)[a] | 5 (4,5)[a] | 4 (1,5)[a] | 6 (5,7)[b] |
| UBC4* | 8 (7,9)[a] | 8 (7,9)[a] | 7 (1,8)[b] | 3 (2,5)[c] |
| UBC6 | 8 (6.5,9)[a] | 6 (4,7)[b] | 7 (6,8.5)[a] | 6 (5,7)[b] |
| UBC9* | 7 (5,8)[a] | 7 (6,8)[a] | 6 (5,7)[b] | 4 (0,5)[c] |
| UBC12* | 7 (5,8)[a] | 6 (4,8)[a] | 5 (6,4)[b] | 4 (2,5)[c] |
| UBC16 | 8 (6,9)[a] | 8 (6,9)[a] | 7 (8.5,6)[a,b] | 7 (6,8)[b] |

*For these primers both contaminated sites showed a higher number of bands than either non-contaminated site.

**For these primers both contaminated sites showed a lower number of bands than either of the non-contaminated sites.

Table 3 - Kendall correlation coefficients and probability values between number of RAPD bands and fecundity for mosquitofish from a radionuclide-contaminated population (Pond 3513). The RAPD banding patterns were produced by several different oligonucleotide primers. Primers were listed here only if $P < 0.10$. For all other primers, $P>0.10$ n (sample size) = 40

| Primer | Coefficient | Probability value |
|---|---|---|
| OPD7 | 0.243 | 0.03 |
| OPD8 | 0.207 | 0.08 |
| OPD13 | 0.207 | 0.08 |
| UBC6* | 0.324 | 0.01 |
| UBC9* | 0.350 | 0.01 |
| UBC12* | 0.394 | <0.01 |

*For these primers, there was a higher average number of bands in the two contaminated populations than in the two non-contaminated populations.

Table 4 - Molecular lengths (in number of nucleotide bases) of 136 RAPD DNA fragments (bands). OPD = Operon Technology Primers, UBC = University of British Columbia Primers.

| | Molecular Length (Number of Nucleotide Bases per Fragment) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **A. OPD Primers** | | | | | | | | |
| Band # | OPD2 | OPD7 | OPD8 | OPD11 | OPD12 | OPD13 | OPD18 | OPD20 |
| 1 | 1586 | 1721 | 1900 | 2566 | 3392 | 899 | 1543 | 1968 |
| 2 | 1399 | 1390 | 1190 | 1826 | 2813 | 817 | 1259 | 1587 |
| 3 | 1196 | 1149 | 1047 | 1501 | 2174 | 787 | 1063 | 1364 |
| 4 | 1060 | 1066 | 881 | 1253 | 1992 | 677 | 951 | 1180 |
| 5 | 990 | 990 | 784 | 976 | 1458 | 595 | 785 | 956 |
| 6 | 664 | 920 | 728 | 833 | 1212 | 493 | 702 | 814 |

Table 4 (cont')

**A. OPD Primers**

| Band # | OPD2 | OPD7 | OPD8 | OPD11 | OPD12 | OPD13 | OPD18 | OPD20 |
|---|---|---|---|---|---|---|---|---|
| 7 | 622 | 856 | 645 | 772 | 1020 | 428 | 602 | 744 |
| 8 | 554 | 743 | 560 | 615 | 753 | 380 | 427 | 605 |
| 9 | 389 | 649 | 438 | 550 | 598 | 313 | 346 | 551 |
| 10 | --- | 480 | --- | 439 | 523 | 263 | --- | 433 |
| 11 | --- | 354 | --- | --- | --- | --- | --- | --- |
| 12 | --- | 276 | --- | --- | --- | --- | --- | --- |

**B. UBC Primers**

| Band # | UBC1 | UBC2 | UBC4 | UBC6 | UBC9 | UBC12 | UBC16 |
|---|---|---|---|---|---|---|---|
| 1 | 1438 | 1305 | 2055 | 1621 | 921 | 1483 | 1594 |
| 2 | 1157 | 1134 | 1610 | 1344 | 804 | 1370 | 1415 |

Table 4 (cont')

**B. UBC Primers**

| Band # | UBC1 | UBC2 | UBC4 | UBC6 | UBC9 | UBC12 | UBC16 |
|---|---|---|---|---|---|---|---|
| 3 | 899 | 908 | 1407 | 1121 | 699 | 1269 | 1100 |
| 4 | 824 | 837 | 1212 | 955 | 625 | 1158 | 1016 |
| 5 | 691 | 803 | 1150 | 799 | 545 | 913 | 814 |
| 6 | 652 | 636 | 687 | 717 | 387 | 861 | 673 |
| 7 | 570 | 569 | 608 | 643 | 299 | 800 | 608 |
| 8 | 514 | --- | 635 | 575 | 187 | 632 | 495 |
| 9 | 440 | --- | 495 | 455 | --- | 449 | 445 |
| 10 | --- | --- | 440 | 385 | --- | 279 | 374 |
| 11 | --- | --- | 390 | --- | --- | --- | --- |
| 12 | --- | --- | 283 | --- | --- | --- | --- |

Table 5 - Frequency of selected RAPD bands* for two radionuclide-contaminated (Pond 3513 and White Oak Lake) and two non-contaminated (Crystal Springs and Wolf Creek) mosquitofish populations. The sample sizes for each population are as follows: Pond 3513, 40; White Oak Lake, 40; Crystal Springs, 38; Wolf Creek, 35.

| | Population | | | |
|---|---|---|---|---|
| Band | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
| $OPD2_{1586}$** | $1.00^a$ | $0.97^a$ | $0.06^b$ | $0.57^c$ |
| $OPD2_{1060}$** | $1.00^a$ | $1.00^a$ | $0.09^b$ | $0.00^c$ |
| $OPD7_{1721}$** | $0.31^a$ | $0.18^a$ | $0.00^b$ | $0.02^b$ |
| $OPD7_{1390}$** | $0.62^a$ | $0.36^b$ | $0.21^c$ | $0.20^c$ |
| $OPD8_{560}$† | $0.85^a$ | $0.67^b$ | $0.93^c$ | $0.97^c$ |
| $OPD13_{787}$† | $0.26^a$ | $0.21^a$ | $1.00^b$ | $0.97^b$ |
| $OPD13_{677}$† | $0.48^a$ | $0.53^a$ | $0.83^b$ | $0.97^c$ |
| $OPD13_{428}$** | $0.96^a$ | $0.74^b$ | $0.59^c$ | $0.00^d$ |
| $OPD13_{263}$† | $0.02^a$ | $0.00^a$ | $0.83^b$ | $0.97^b$ |
| $OPD20_{605}$** | $0.20^a$ | $0.67^b$ | $0.06^c$ | $0.11^c$ |
| $OPD20_{433}$** | $0.78^a$ | $0.48^b$ | $0.09^c$ | $0.00^d$ |

Table 5 (cont')

| Band | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|---|
| $UBC4_{2055}$** | $0.91^a$ | $0.74^b$ | $0.52^c$ | $0.10^d$ |
| $UBC4_{1610}$** | $0.71^a$ | $0.77^a$ | $0.31^b$ | $0.27^b$ |
| $UBC4_{1407}$** | $0.47^a$ | $0.53^a$ | $0.34^b$ | $0.13^c$ |
| $UBC6_{575}$** | $0.44^a$ | $0.64^b$ | $0.21^c$ | $0.31^c$ |
| $UBC6_{455}$** | $0.20^a$ | $0.23^a$ | $0.00^b$ | $0.00^b$ |
| $UBC9_{921}$** | $0.77^a$ | $0.52^b$ | $0.45^c$ | $0.03^d$ |
| $UBC12_{1269}$** | $0.57^a$ | $0.48^b$ | $0.00^c$ | $0.00^c$ |
| $UBC12_{1158}$** | $0.72^a$ | $0.19^b$ | $0.00^c$ | $0.00^c$ |
| $UBC12_{861}$** | $0.13^a$ | $0.29^b$ | $0.00^c$ | $0.02^c$ |
| $UBC16_{1016}$ | $0.76^a$ | $0.78^a$ | $0.36^b$ | $0.43^b$ |

*Only bands which show consistent differences in bandfrequency between the two contaminated and two non-contaminated populations are reported here.

**Both contaminated sites had a higher frequency of these bands than either of the non-contaminated sites.

†Both contaminated sites had a lower frequency of these bands than either of the non-contaminated sites.

Table 6- Median (+third quartile, -first quartile)* fecundity of fish with and without particular RAPD bands. These fish were collected from a radionuclide-contaminated population (Pond 3513). Numbers in brackets (eg [12]) refer to sample sizes. Fecundity was calculated as brood size/body length of the mother.

| | Median ($^{+TQ}_{-FQ}$) MML | | |
|---|---|---|---|
| Band** | With Band | Without Band | P§ |
| $OPD7_{1721}$ | 1.74 ($^{+0.17}_{-0.28}$) [12] | 1.70 ($^{+0.26}_{-0.59}$) [27] | 0.29 |
| $OPD7_{1390}$ | 1.78 ($^{+0.28}_{-0.16}$) [25] | 1.57 ($^{+0.57}_{-0.14}$) [14] | 0.05 |
| $OPD8_{560}$ | 1.80 ($^{+0.17}_{-0.30}$) [34] | 1.70 ($^{+0.35}_{-0.24}$) [6] | 0.23 |
| $OPD13_{677}$ | 1.74 ($^{+0.22}_{-0.20}$) [10] | 1.68 ($^{+0.20}_{-0.32}$) [29] | 0.15 |
| $OPD13_{595}$ | 1.73 ($^{+0.29}_{-0.27}$) [19] | 1.67 ($^{+0.13}_{-0.32}$) [20] | 0.15 |
| $OPD20_{605}$ | 1.97 ($^{+0.25}_{-0.20}$) [8] | 1.74 ($^{+0.24}_{-0.15}$) [32] | 0.05 |
| $OPD20_{433}$ | 1.81 ($^{+0.19}_{-0.13}$) [31] | 1.64 ($^{+0.25}_{-0.47}$) [9] | 0.05 |
| $UBC4_{2055}$ | 1.71 ($^{+0.21}_{-0.15}$) [36] | 1.81 ($^{+0.19}_{-0.35}$) [4] | 0.34 |
| $UBC4_{1610}$ | 1.71 ($^{+0.60}_{-0.35}$) [27] | 1.81 ($^{+0.19}_{-0.17}$) [11] | 0.07 |
| $UBC4_{1407}$ | 1.71 ($^{+0.28}_{-0.09}$) [18] | 1.72 ($^{+0.17}_{-0.31}$) [20] | 0.26 |
| $UBC6_{575}$ | 1.72 ($^{+0.30}_{-0.35}$) [17] | 1.46 ($^{+0.21}_{-0.75}$) [23] | 0.02 |

Table 6 (cont')

| Band | Median ($^{+TQ}_{-FQ}$) MML With Band | Median ($^{+TQ}_{-FQ}$) MML Without Band | P[§] |
|---|---|---|---|
| $UBC6_{455}$ | 1.75 ($^{+0.27}_{-0.60}$) [8] | 1.57 ($^{+0.42}_{-0.22}$) [32] | 0.08 |
| $UBC9_{921}$ | 1.71 ($^{+0.20}_{-0.12}$) [30] | 1.44 ($^{+0.15}_{-0.70}$) [9] | 0.02 |
| $UBC12_{1269}$ | 1.81 ($^{+0.21}_{-0.13}$) [21] | 1.66 ($^{+0.08}_{-0.30}$) [18] | 0.03 |
| $UBC12_{1158}$ | 1.76 ($^{+0.24}_{-0.09}$) [28] | 1.46 ($^{+0.08}_{-0.34}$) [11] | <0.01 |
| $UBC12_{861}$ | 1.71 ($^{+0.04}_{-0.04}$) [28] | 1.75 ($^{+0.14}_{-0.30}$) [11] | 0.32 |
| $UBC16_{1016}$ | 1.75 ($^{+0.22}_{-0.15}$) [30] | 1.58 ($^{+0.12}_{-0.29}$) [10] | 0.04 |

*Quartiles are a non-parametric measure of variance roughly analogous to standard deviation. I.e., 1/4 of the population have values less than the first quartile and 1/4 have values which are greater than the third quartile.

**This table presents data for 16 of the 20 bands listed in Table 5 for which the frequency was higher in contaminated than non-contaminated sites. Bands $OPD2_{1586}$ and $OPD2_{1060}$ were omitted from Table 6 because all of the fish in Pond 3513 had these bands, so no comparisons could be made between fish with and without these bands. Band $OPD13_{428}$ was omitted because only two individuals did not have this band, and $OPD13_{263}$ was

Table 6 (cont')

omitted because only one individual Pond 3513 had this band. Therefore it was felt that valid statistical tests could not be performed on these bands. All other bands listed in Table 5 are present in Table 6.

§Probability values for the test of $H_o$: no difference between individuals with or without each band (Wilcoxon Rank-Sum Test).

Table 7 - Roger's genetic distance for RAPD genotypes between four different mosqutiofish populations, measured for individual primers. The populations include two contaminated sites - Pond 3513 and White Oak Lake (WOL) - and two non-contaminated sites - Crystal Springs (CS) and Wolf Creek (WC). These distances were calculated from RAPD patterns using oligonucleotide primers. The sample sizes for each population are as follows: Pond 3513, 40; White Oak Lake, 40; Crystal Springs, 38; Wolf Creek, 35. Values with similar superscripts are not significantly different ($P<0.05$, Kruskall-Wallace test).

| | Distance | | | | | |
|---|---|---|---|---|---|---|
| Primer | 3513-CS | 3513-WOL | 3513-WC | CS-WOL | CS-WC | WOL-WC |
| OPD2 | $0.78^{a}$ | $0.97^{b}$ | $0.87^{a}$ | $0.78^{a}$ | $0.85^{a}$ | $0.88^{a}$ |
| OPD7 | $0.70^{a}$ | $0.69^{a}$ | $0.67^{a}$ | $0.60^{b}$ | $0.61^{b}$ | $0.57^{b}$ |
| OPD8 | $0.15^{a}$ | $0.24^{a}$ | $0.11^{a}$ | $0.19^{a}$ | $0.121^{a}$ | $0.22^{a}$ |
| OPD11 | $0.25^{b}$ | $0.24^{b}$ | $0.54^{c}$ | $0.19^{a}$ | $0.52^{c}$ | $0.44^{c}$ |
| OPD12 | $0.21^{a}$ | $0.08^{b}$ | $0.14^{a}$ | $0.19^{a}$ | $0.29^{c}$ | $0.21^{a}$ |
| OPD13 | $0.25^{a}$ | $0.08^{b}$ | $0.33^{a}$ | $0.28^{a}$ | $0.12^{c}$ | $0.33^{a}$ |
| OPD18 | $0.20^{a}$ | $0.28^{c}$ | $0.36^{c}$ | $0.21^{b,c}$ | $0.50^{d}$ | $0.36^{c}$ |
| OPD20 | $0.21^{b}$ | $0.15^{a}$ | $0.30^{c,d}$ | $0.24^{b}$ | $0.28^{b,c}$ | $0.35^{d}$ |
| UBC1 | $0.13^{a}$ | $0.37^{b}$ | $0.36^{b}$ | $0.38^{b}$ | $0.39^{b}$ | $0.32^{b}$ |

Table 7 (cont')

| Primer | 3513-CS | 3513-WOL | 3513-WC | CS-WOL | CS-WC | WOL-WC |
|---|---|---|---|---|---|---|
| UBC2 | $0.26^{a}$ | $0.26^{a}$ | $0.27^{a}$ | $0.12^{b}$ | $0.35^{c}$ | $0.40^{c}$ |
| UBC4 | $0.23^{b}$ | $0.14^{a}$ | $0.60^{d}$ | $0.14^{a}$ | $0.42^{c}$ | $0.52^{c}$ |
| UBC6 | $0.20^{a,b}$ | $0.28^{a,b}$ | $0.26^{b}$ | $0.23^{a,b}$ | $0.18^{a}$ | $0.26^{b}$ |
| UBC9 | $0.11^{a}$ | $0.12^{b}$ | $0.27^{c}$ | $0.13^{d}$ | $0.28^{c}$ | $0.22^{c}$ |
| UBC12 | $0.28^{a}$ | $0.31^{a}$ | $0.24^{a}$ | $0.21^{a}$ | $0.15^{b}$ | $0.31^{a}$ |
| UBC16 | $0.18^{a}$ | $0.20^{a}$ | $0.21^{b}$ | $0.24^{c}$ | $0.15^{d}$ | $0.16^{a}$ |

Table 8 - Roger's genetic distance for RAPD genotypes between four different mosqutiofish populations, averaged over all RAPD primers. The populations include two contaminated sites - Pond 3513 and White Oak Lake - and two non-contaminated sites - Crystal Springs and Wolf Creek. Values with the same superscripts are not significantly different ($P<0.05$, Kruskall-Wallace test)

| | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|
| Pond 3513 | $0.29^{a}$ | $0.28^{a}$ | $0.37^{b}$ |
| White Oak Lake | --- | $0.28^{a}$ | $0.37^{b}$ |
| Crystal Springs | --- | --- | $0.35^{b}$ |

Table 9 - Roger's genetic distance for allozyme genotypes between four different mosqutiofish populations. The populations include two contaminated sites - Pond 3513 and White Oak Lake - and two non-contaminated sites - Crystal Springs and Wolf Creek. Distances were calculated using all loci (upper right) or the nucleoside phosphorylase locus only (lower left). Values with the same uppercase or lowercase letters are not significantly different ($P<0.05$, Kruskall-Wallace test)

| | Pond 3513 | White Oak Lake | Crystal Springs | Wolf Creek |
|---|---|---|---|---|
| Pond 3513 | --- | $0.036^{A}$ | $0.014^{B}$ | $0.015^{B}$ |
| White Oak Lake | $0.035^{a}$ | --- | $0.031^{A}$ | $0.025^{C}$ |
| Crystal Springs | $0.115^{b}$ | $0.080^{c}$ | --- | $0.054^{D}$ |
| Wolf Creek | $0.068^{d}$ | $0.040^{b}$ | $0.047^{c}$ | --- |

**Figure Legends**

Figure 1 - Hypothetical gel showing RAPD banding patterns. The bands are numbered from highest molecular length to lowest. Notice that in this sample, there are three genotypes and five individuals.

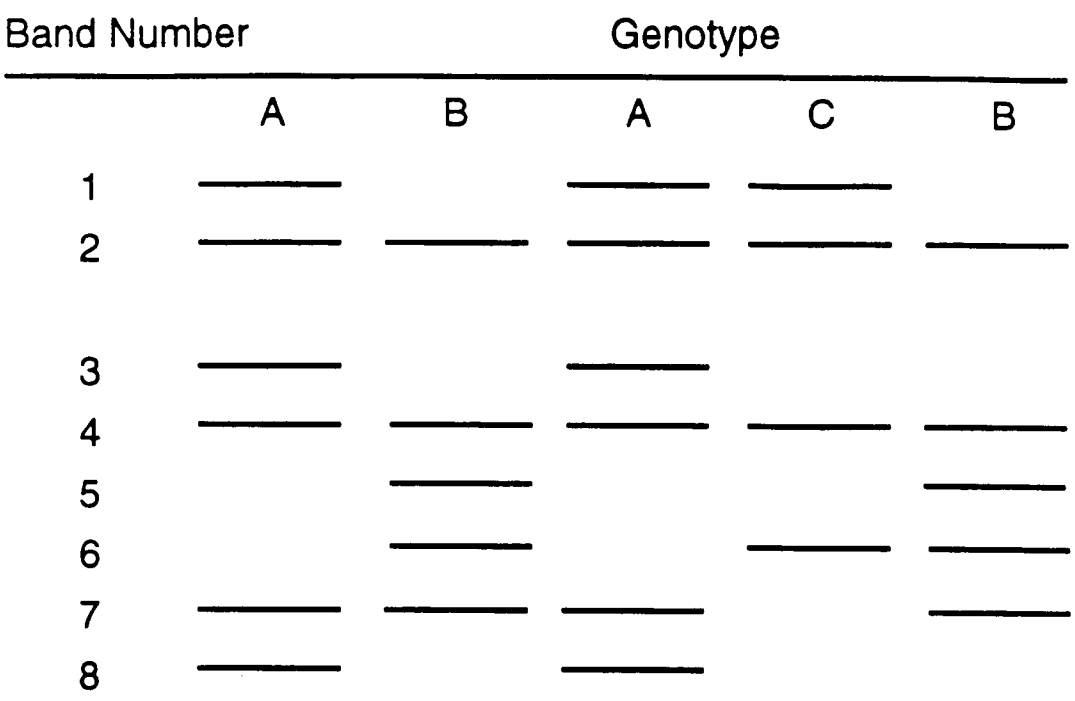
Figure 2 - Number of RAPD bands for homozygotes and heterozygotes as determined by allozyme analysis (heterozygote = heterozygous for at least one locus, homozygous = homozygous for all loci). These data were obtained from a radionuclide-contaminated population (Pond 3513). The values presented here represent medians with first and third quartiles. The numbers above each pair of bars represent the significance levels for the test of Ho: no difference between homozygotes and heterozygotes (Wilcoxon rank-sum test). The number of heterozygotes and homozygotes were 18 and 22, respectively.

Figure 3 - Correlation between frequency of 17 RAPD bands vs. selection coefficient for such bands. The selection coefficient = 1 - w, where w, the relative fitness of individuals not possessing these bands, is equal to the fecundity of individuals without each band/ the fecundity of individuals with these bands. The frequency was calculated at number of fish with this band ÷ the total number of fish in the population.

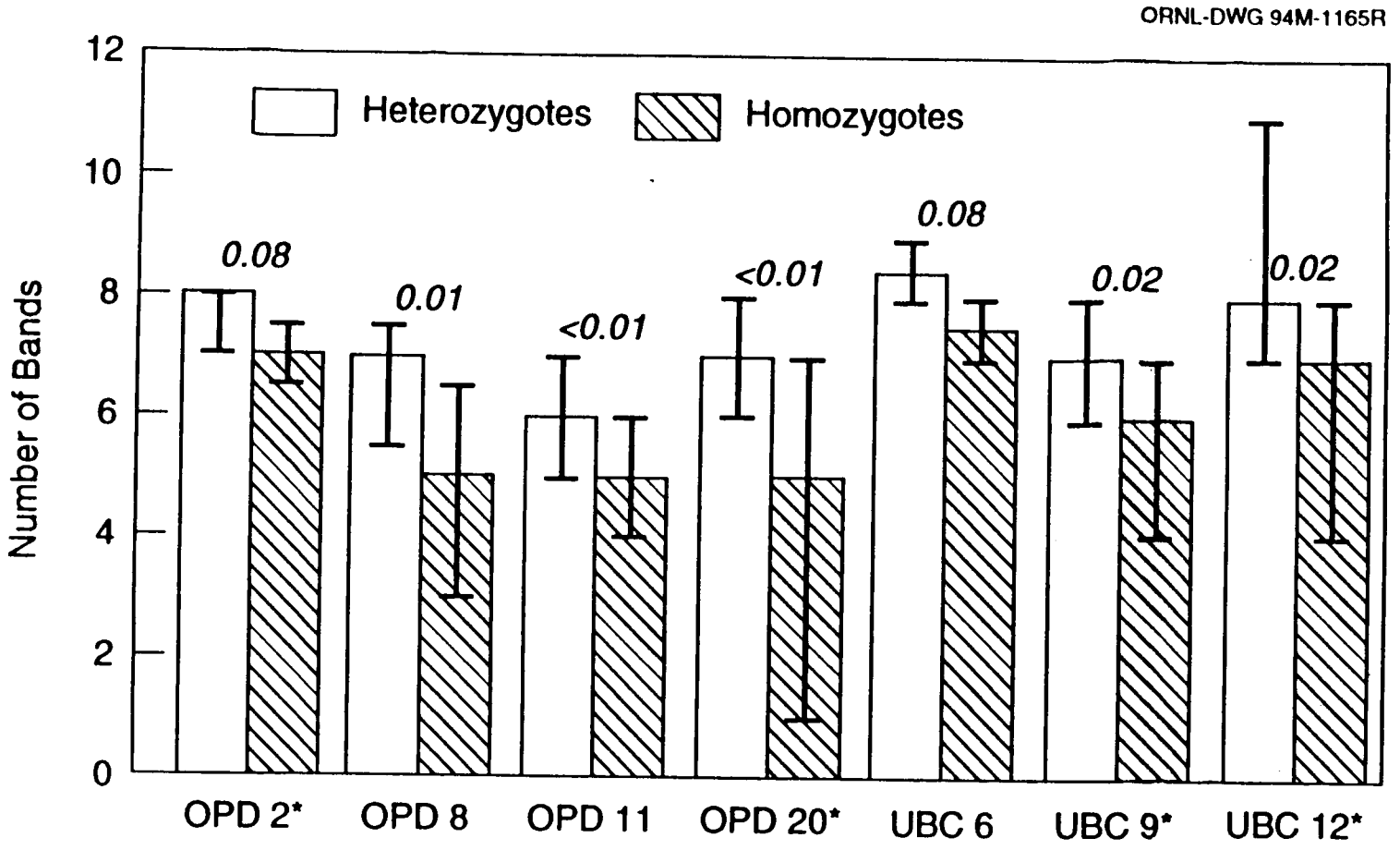
Figure 4 - Allozyme heterozygosity and percent polymorphism in mosquitofish populations. A) Percent polymorphism as determined by allozyme analysis. Bars labeled with the same letter are not statistically different ($P<0.05$, $\chi^2$ test). B) Average heterozygosity as determined by allozyme electrophoresis. The heterozygosity of nucleoside phosphorylase (NP) and all other loci were analyzed separately. Bars labeled with the same uppercase (NP) or lowercase (all other loci) letters are not statistically different ($P<0.05$, Kruskall-Wallace test). Data are presented for two radionuclide-contaminated (Pond 3513 and White Oak Lake) and two non-contaminated (Crystal Springs and Wolf Creek) populations. Sample sizes for each population were as follows: Pond 3513, 40; White Oak Lake; 40: Crystal Spring, 38; Wolf Creek, 35.

Figure 5 - Fecundity of heterozygous and homozygous mosquitofish from a radionuclide-contaminated pond (Pond 3513). Heterozygosity was determined for the nucleoside phosphorylase (NP) locus and for all loci separately. Sample sizes for heterozygotes and homozygotes for the NP locus were 14 and 26, respectively, and for all other loci were 6 and 34, respectively.

Figure 6 - Frequency of different nucleoside phosphorylase genotypes in two radionuclide-contaminated (White Oak Lake and Pond 3513) and two non-contaminated (Crystal Springs and Wolf Creek) mosquitofish populations. For each genotype, bars labeled with different letters are significantly different ($P<0.05$, Kruskall-Wallace test). F = fast allele, S = slow allele, relative to migrational speed during starch gel electrophoresis.




Number of genotypes: 3
Average number of bands: 5.2
Genotype frequency: A = 0.4; B = 0.4; C = 0.2
Frequency of band 1: 0.6
Frequency of band 2: 1.0
Frequency of band 3: 0.4


ORNL-DWG 94M-1165R
Heterozygotes
Homozygotes
Number of Bands
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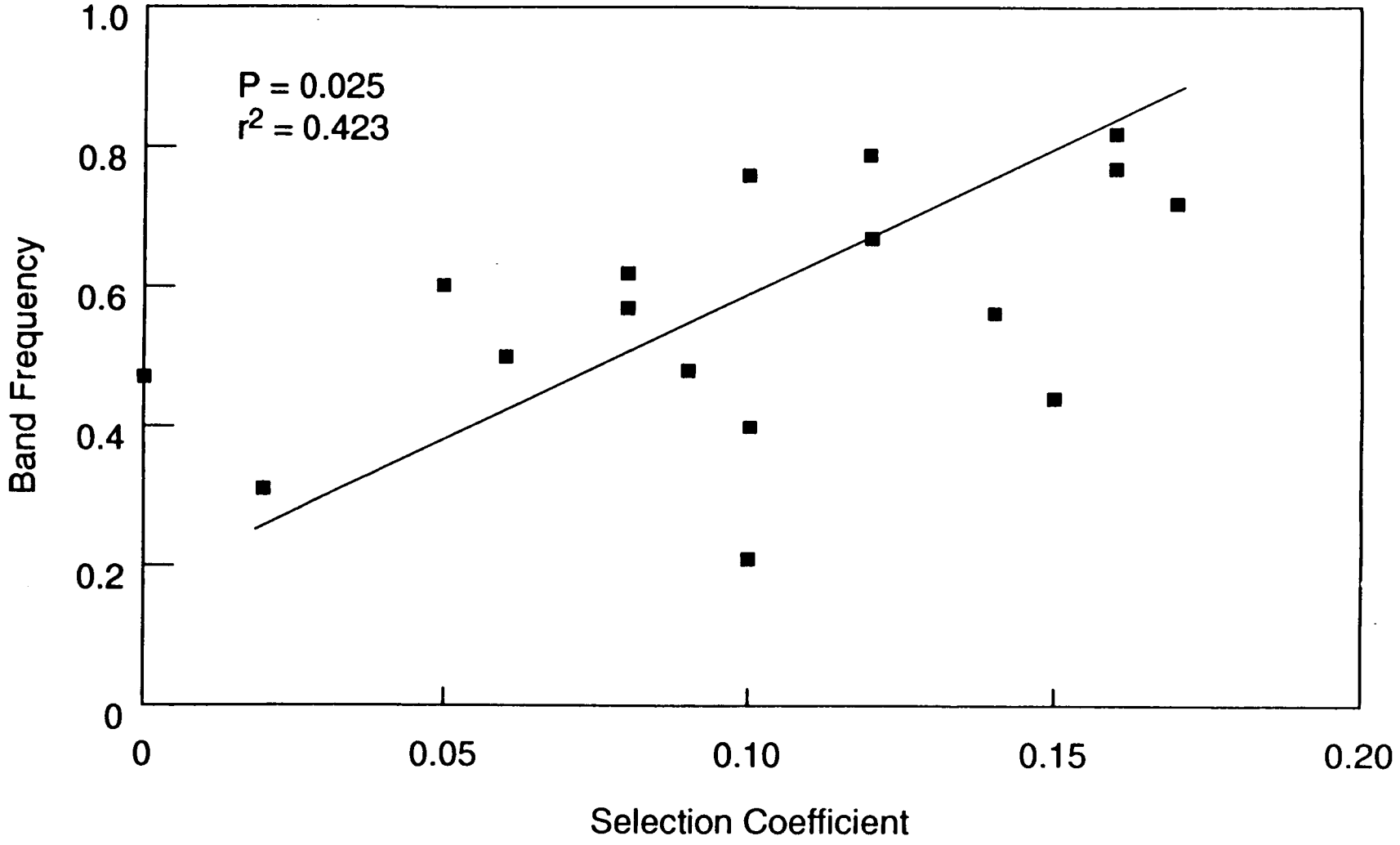
10
8
6
4
2
0
0.08
0.01
<0.01
<0.01
0.08
0.02
0.02
OPD 2*
OPD 8
OPD 11
OPD 20*
UBC 6
UBC 9*
UBC 12*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r2 = 0.423
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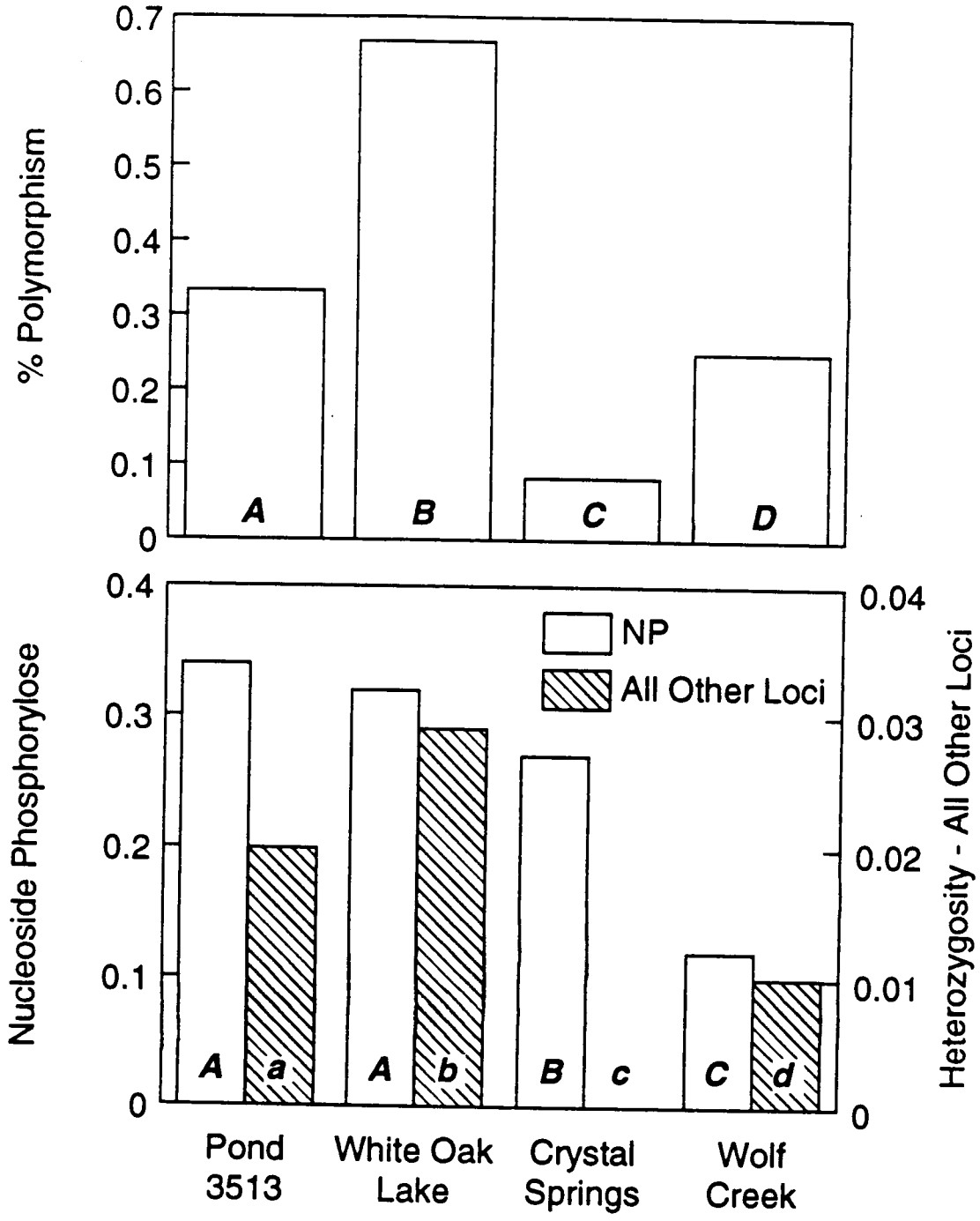
0.8
0.6
0.4
0.2
0
0
0.05
0.10
0.15
0.20
Band Frequency
Selection Coefficient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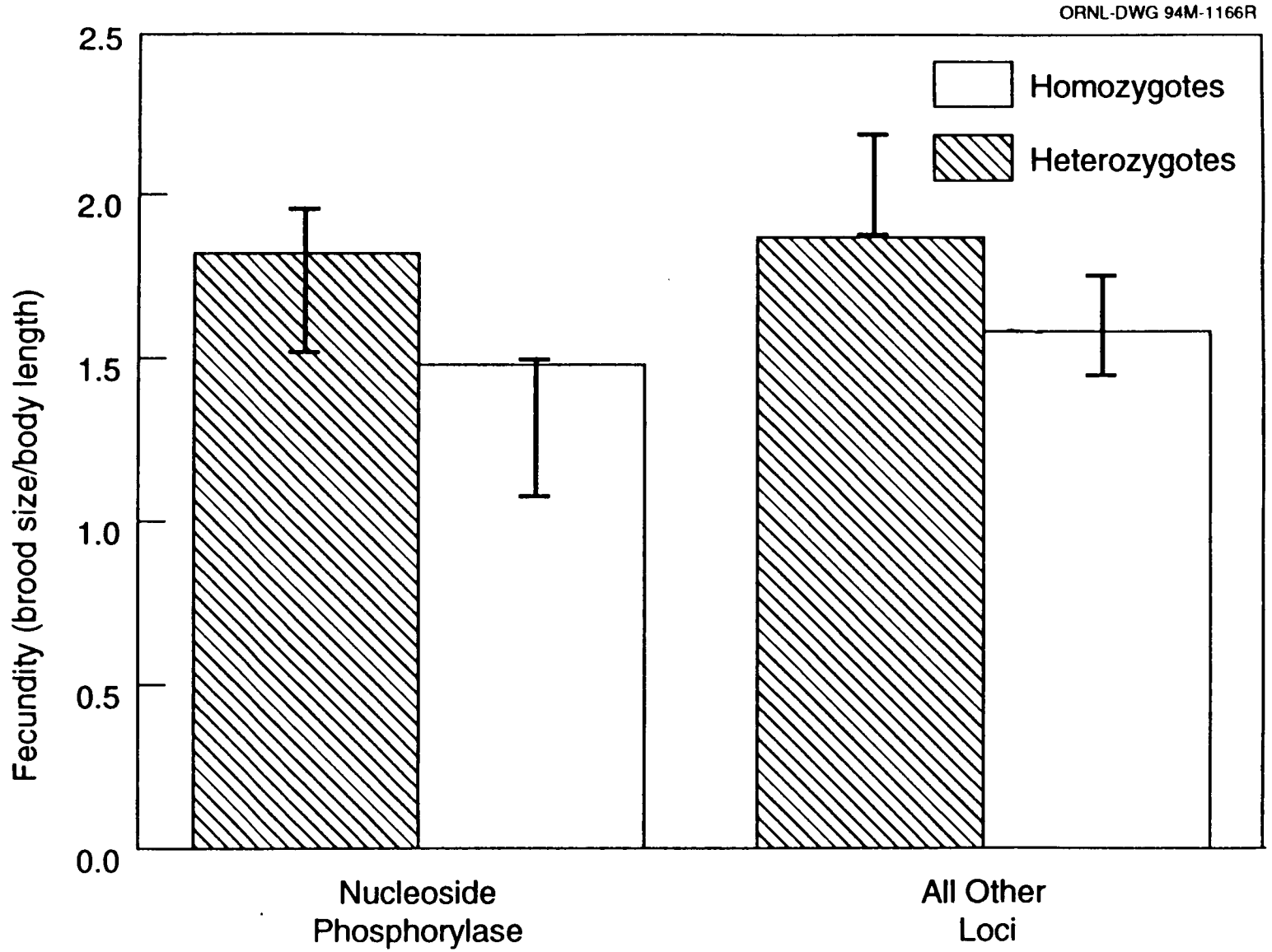
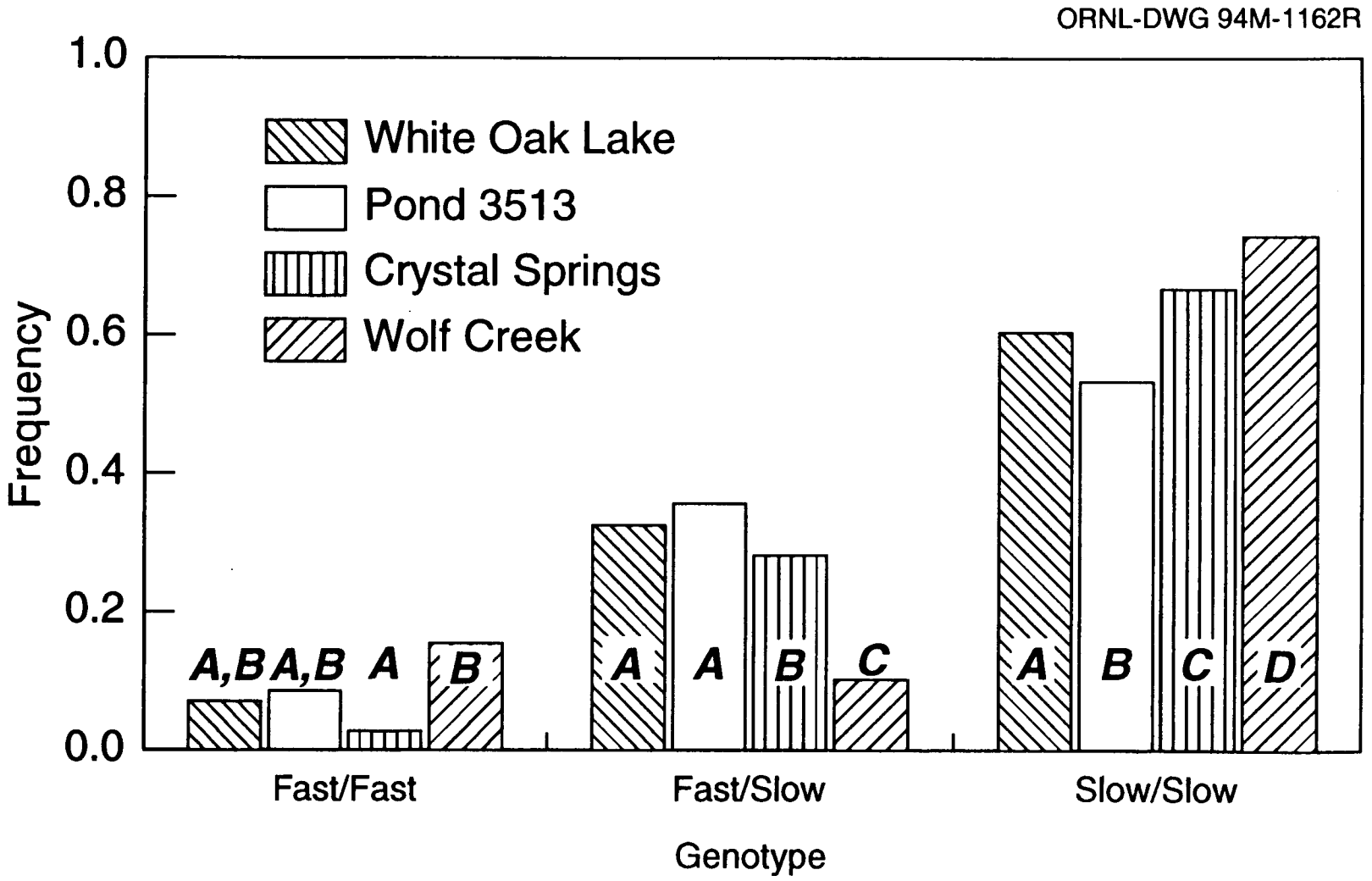
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
B
C
D
Nucleoside Phosphorylose
Heterozygosity - All Other Loci
0.4
0.3
0.2
0.1
0
0.04
0.03
0.02
0.01
0
NP
All Other Loci
A
a
A
b
B
c
C
d
Pond 3513
White Oak Lake
Crystal Springs
Wolf Creek


ORNL-DWG 94M-1166R
Homozygotes
Heterozygotes
Fecundity (brood size/body length)
2.5
2.0
1.5
1.0
0.5
0.0
Nucleoside Phosphorylase
All Other Loci


ORNL-DWG 94M-1162R
1.0
0.8
0.6
0.4
0.2
0.0
Frequency
White Oak Lake
Pond 3513
Crystal Springs
Wolf Creek
A,B
A,B
A
B
A
A
B
C
A
B
C
D
Fast/Fast
Fast/Slow
Slow/Slow
Genotype

# PART 6

# GENETIC ECOTOXICOLOGY OF RADIONUCLIDES IN MOSQUITOFISH. III: THE RELATIONSHIP BETWEEN DNA STRAND BREAKS AND GENOTYPE

## JUSTIFICATION

In chapter 3 it was found that exposure to radionuclides may lead to DNA strand breakage, and that there was a correlation between fecundity and strand breakage, suggesting that resistance to strand breakage carries a fitness component. This also suggests that certain genotypes may confer greater DNA strand breakage resistance or repair, thus conferring a selective advantage to these genotypes. This hypothesis was supported by the findings in chapter 3 which suggested that exposure to radionuclides may alter population genetic structure. Therefore this chapter will address the question as to whether or not there is a relationship between the degree of strand breakage and genotype.

**ABSTRACT**

DNA polymorphisms revealed by RAPD and allozyme analysis were compared to relative amounts of strand breakage as determined by gel electrophoresis in mosquitofish (Gambusia affinis). These fish were exposed to radionuclide contamination in the field and to X-rays in the laboratory. The types of RAPD metrics used were number of bands per individual and frequency of certain bands. It was found in a previous study that in some instances the number of bands and the frequency of certain bands in the population were elevated in radionuclide-contaminated sites relative to reference sites. In several cases, the median molecular length (MML) of the DNA (which is inversely proportional to the amount of strand breakage) was correlated to the number of bands per individual. Also, for bands which had a higher population frequency in contaminated sites, the MML in many cases was higher for fish with than without these bands. Results from laboratory X-ray exposures paralleled those from the field.

## INTRODUCTION

It is generally accepted that the phenotype of an organism is adapted for the ambient environmental conditions in which it lives. Because the phenotype displayed by an organism is at least partly determined by the genotype, it follows that there should be genotypes which are more adapted to certain environments than others. In many cases, genotypic differences between the same species living under different environmental conditions have been found (eg., see Merrill 1981 for a review and discussion). These differences can be assigned an adaptive value only if they provide some sort of advantage under certain conditions (Koehn and Hilbish 1987) and/or are inexplicable on the basis of neutrality theory (Hartl and Clark 1989). For example, differences in the kinetic properties of lactate dehydrogenase (LDH) and aminopeptidase allozymes of fish and mollusks _in vitro_ paralleled physiological differences _in vivo_. Both were in accordance with differences in allele frequencies and environmental variables in the field (Koehn et al. 1985, Powers et al. 1983).

It has also been found that organisms may respond to anthropogenic chemicals in a similar fashion. For instance, it has been found that allele and genotype frequencies in fish differ between contaminated and reference sites, and this corresponds to differences in heavy metal tolerance in the

laboratory (Gillespie and Guttman 1988). Further studies have shown that genotypic differences of heavy metal tolerance *in vivo* parallel differences in allele-specific enzyme inhibition by heavy metals *in vitro* (Changon and Guttman 1989a, 1989b). Thus it seems that certain genotypes have an advantage over others in contaminated populations, and that this advantage may have a physiological basis. Most of these studies have focused on the effects of heavy metals, while other types of pollution have gotten less attention. Another type of environmental contaminant includes genotoxins: materials which alter the structure and function of DNA. To our knowledge, there has not been any work done on the interaction between genotype and genotoxicant exposure.

Additionally, most of the laboratory studies linking genotype with phenotypic responses to contaminants have focused on mortality as an endpoint of contaminant exposure. Not much work has been done using biomarkers (biological indicators of sublethal contaminant exposure) as phenotypes in population genetic studies. Sublethal effects deserve consideration because ecological effects of contamination may be due to sublethal as well as lethal responses of organisms to pollution. One biomarker of genotoxic exposure in fish is DNA strand breakage (Shugart 1988). In particular, it has been found that mosquitofish (*Gambusia affinis*) living in radionuclide-contaminated environments have a higher level of strand breakage than do fish in reference sites. Furthermore,

within one contaminated site (Pond 3513), the level of strand breakage is negatively correlated with fecundity (see Chapter 3), suggesting that genotypes which confer resistance to genotoxicity may have a selective advantage over others in the population. Also, it has been found that certain RAPD and allozyme genotypes are more common in the contaminated sites than in the non-contaminated sites, and that fish in Pond 3513 with the common genotypes are more fecund than those with the less common genotypes (see chapter 4). It therefore follows that there may be genotypic differences in the level of DNA strand breakage when mosquitofish are exposed to ionizing radiation. Such differences in strand breakage may depend on genetic predisposition to resist or repair DNA damage. Thus, in a sense the relative levels of strand breakage within the population may be thought of as phenotypic variables.

Therefore the objective of this study is to determine the relationship between a phenotypic response - in this case DNA strand breakage, a biomarker of genotoxic exposure - and genotype in irradiated fish. This objective will be accomplished using mosquitofish and RAPD and allozyme techniques. One part of the study involves examining this relationship in a radionuclide-contaminated pond in the field. The second part of the study involves exposing the fish to X-rays in the laboratory. X-rays were used because logistical constraints precluded the use of a $\gamma$-radiation source in the laboratory. It is hypothesized that fish with genotypes which

are more common in the contaminated environments will have a lower level of strand breakage - both in the field and in the laboratory - than fish with the less common genotypes. It had been found in a previous study that RAPD genotypes may differ between contaminated and non-contaminated populations. Two principal metrics used to qualify differences in RAPD genotype were average number of bands per individual and frequency of certain bands (see chapter 4). Therefore the following predictions are made: 1) Individuals which exhibit a greater number of RAPD bands should be more resistant to strand breakage 2) Some bands appear more frequently in fish in contaminated than non-contaminated site (see chapter 4). Thus individuals which produce such bands should have a lower number of strand breaks than those that do not. 3) Nucleoside phosphorylase (NP) heterozyotes are increased in frequency in contaminated sites (see chapter 4). Therefore NP heterozygotes should exhibit fewer strand breaks than other genotypes.

## METHODS AND MATERIALS

### Specimen Collection

Adult female mosquitofish were collected by dipnet from Pond 3513, which is heavily contaminated with $^{235}Pu$, $^{37}Cs$, $^{90}Sr$ and other radionuclides (Tamura et al. 1977). For a detailed description of the site, see Part 5.

For laboratory exposures, adult female fish were collected from a small pond on the ORNL grounds which is not known to be contaminated with radionuclides. The fish were kept in flowing well water and fed freeze-dried Tubificid worms ad libitum for at least one week prior to the start of the experiments.

### Sample Analysis

For the Pond 3513 specimens, blood and liver tissues and carcasses (head and internal organs removed) were collected as described in chapter 4. For the laboratory exposures, specimens were sacrificed by spinal scission immediately after X-ray exposure and liver tissues and carcasses were immediately frozen in liquid nitrogen and stored at -70° C until analysis.

DNA was extracted from blood (field samples only) and liver tissues as described previously (see chapter 3).

Carcasses were used for allozyme analysis and DNA for RAPD analysis as described in chapter 3. The terminology for naming RAPD genotypes was discussed in chapter 4. The particular primers and bands used for this study were chosen because they showed differences between contaminated and non-contaminated populations either in terms of number of RAPD bands per individual or frequency of certain bands in each population (the banding patterns produced by primer UBC9, for example, displayed higher numbers of bands in individuals living in contaminated sites, and a higher frequency of band $UBC9_{921}$ in contaminated sites [see Part 5]).

DNA strand breakage was determined electrophoretically as described in Part 4. Briefly, the extracted DNA sample consists of DNA fragments of various sizes. The sample was analyzed via gel electrophoresis and the midpoint of the DNA size distribution in the gel was determined by densitometry. This midpoint is referred to as the median molecular length (MML). The MML is inversely proportional to the number of strand breaks: the more strand breaks, the smaller the DNA fragments and the lower the MML. Electrophoresis was performed at alkaline (pH 12) conditions to examine total (both single and double) strand breaks and under neutral (pH 7) conditions to examine double-strand breaks only. Because the MML of liver DNA was different than that of blood (see chapter 3), both tissues were examined for comparison.

Some RAPD bands were present at a higher frequency in the contaminated than non-contaminated sites (see Part 5). For these bands the MML difference was calculated as MML of fish with minus the MML of fish without these bands (i.e., relative advantage of possessing these bands). The MML differences were compared to the frequency of these bands in the Pond 3513 population by Kendall's test of correlation.

Further, it was found in chapter 4 that there were differences in fecundity and genotype frequencies for the nucleoside phosphorylase (NP) locus, so the MML of different NP genotypes also were compared.

**X-ray exposure**

Fish were exposed in 1 cm well water in a 10 cm diameter glass examination dish. The source of the X-rays was a G.E. Maximar 250 III apparatus. This apparatus was set at 250 kV, 15 mA, and gave a dose rate of 1.2 Gy/min at 40 cm. The dose rate was measured with a Victoreen 250R condenser-type pocket ionization chamber. During exposure, the glass examination plate was constantly rotated on a revolving platform for uniform exposure. Preliminary experiments suggested that a dose of 20 Gy was sufficient to produce the appropriate amount of strand breakage. The fish were sacrificed immediately following exposure. Because DNA repair is initiated relatively rapidly in fish following X-ray exposure (Shugart

1989), collection of blood was omitted in order to collect and freeze liver tissue as quickly as possible.

## RESULTS

### RAPD Analysis

Field study - There were statistically significant positive correlations ($P \leq 0.05$) between number of bands and MML for primers UBC9 and UBC16 (liver), UBC12 (blood), UBC12 (blood), and UBC6 (liver and blood) - i.e., fish which displayed more bands had fewer strand breaks (higher MML). There were correlations which were significant at 0.10 but not at 0.05 for primers UBC4 (liver), OPD7 (blood), and OPD20 (liver and blood; Table 1[5]).

There also differences in MML between fish with and without certain bands. Table 2 presents data for the bands from Part 5 for which there were significant differences in band frequency between contaminated and non-contaminated sites (See Part 5, Table 4). There were significant differences in MML at the 0.05 level (Wilcoxon rank-sum test) between fish with and without these bands. There were a total of 56 comparisons made: 4 comparisons for each of the 14 primers (total strand breaks in liver and blood, double strand breaks in liver and blood). Of these 56, there were 20 comparisons for which the differences were significant at the 0.05 level. There also were an additional 9 comparisons that were significant at the 0.10 level (Table 2). There were some

[5]All tables and figures to Part 6 are found in the appendix to Part 6.

differences between total and double strands breaks, i.e, the difference was significant at the 0.10 level for total but not double strand breaks or vice versa. This was true for bands $OPD7_{1721}$ and $OPD20_{433}$ (for blood DNA); $OPD13_{677}$, $UBC4_{1610}$, $UBC4_{1407}$, and $UBC12_{1158}$ (for liver DNA); $OPD7_{1390}$ and $UBC4_{1407}$ (for liver and blood DNA). There also were some cases for which the differences were significant for liver but not blood DNA. This was true for bands $OPD7_{1721}$ , $OPD13_{677}$, $UBC4_{1610}$, $UBC4_{1407}$, and $UBC6_{455}$ (total strand breaks); $UBC12_{1269}$ (double strand breaks); $OPD7_{1390}$, $OPD20_{433}$, and $UBC12_{1158}$ (total and double strand breaks).

For primer UBC4 there was a negative correlation (P = 0.06) between number of bands and MML (Table 1): fish with more of these bands seemed to have more strand breaks. Furthermore, fish with bands $UBC4_{1610}$ and $UBC4_{1407}$ had more strand breaks than fish without these bands.

The MML difference (i.e. relative advantage of possessing these bands) was positively correlated with the frequency of these bands in the Pond 3513 population (Fig. 1).

Laboratory Study - The results from laboratory exposures of Gambusia generally paralleled those from the field. For 3 of the 6 primers in Table 3 the correlations between number of bands and MML were significant at 0.05, and there was one additional correlations significant at 0.10. In comparing the MML for fish with and without specific bands, it was found

that there were 5 bands out of 11 for which the differences were significant at the 0.05 level and one additional band for which the difference was significant at 0.10 (Table 4). However, there were a few discrepancies between laboratory and field data. In 3 cases there were significant (at $P \leq 0.05$ or 0.10) correlations in the laboratory (i.e., liver DNA) but not in liver DNA from the field (or vice versa) for total and/or double strand breaks (see primers OPD7, UBC9, and UBC12). In terms of differences in MML between fish with and without particular bands, discrepancies between field and laboratory fish existed for bands $OPD7_{1721}$, $UBC4_{1610}$, $UBC6_{575}$, $UBC9_{921}$, and $UBC12_{1269}$.

The bands presented in Table 4 were chosen because the were present at a higher frequency in contaminated sites relative to reference sites (see Part 5). Some of the bands which were found at a higher frequency in contaminated relative to reference sites and were presented in Part 5 and/or Table 3 are not represented in Table 4. This is because these bands were absent in fish from the population used in the laboratory studies. These include $OPD2_{1586}$, $OPD2_{1060}$, $OPD13_{428}$, $UBC4_{440}$, $UBC6_{455}$, $UBC12_{1158}$ and $UBC12_{861}$.

**Allozyme Analysis**

For liver DNA, there were no differences between the FF and FS genotypes for total strand breakage, but both were

greater than SS. In the blood, the order of increasing MML was SS < FF < FS. No differences were found for double strand breaks only (Fig. 2). These differences were found for total strand breaks, but no differences were found for double-strand breaks.

# DISCUSSION

The results of this study suggest that there may be a relationship between RAPD or allozyme genotype and the degree of strand breaks. These patterns were found in terms of correlations between number of RAPD bands and strand breaks, differences in strand breaks between fish with and without specific bands and between nucleoside phosphorylase genotypes. These findings support the hypotheses and predictions stated earlier. This is in accordance with other studies which have shown that there are differences in genotype between contaminated and non-contaminated populations, that these differences in many case reflect genotypic differences in fecundity in Pond 3513, and that there were correlations between MML and fecundity in Pond 3513 (chapters 3 and 4). This type of finding is important in establishing the usefulness of RAPD genotype as an indicator of genotoxic exposure. In particular, this is one of the first studies attempting to link genotypic responses with biomarker responses. Such linkages may be important in determining the physiological or ecological significance of altered genetic structures in populations.

## RAPD Analysis

The results of this study support the hypothesis that the response of individuals to DNA damage was related to genotype. In general, it was found that fish with RAPD banding patterns which were more common in contaminated environments had lower levels of strand breakage both in the field and in the laboratory. This supports the original hypothesis that fish with banding patterns which were common in radionuclide-contaminated sites should have a lower number of strand breaks (i.e., a higher MML) than the others in the population. If these bands provide an adaptive advantage to mosquitofish in terms of DNA strand break resistance or repair, one would expect that the relative advantage of having these bands would be proportional to their frequency in the population. The data presented here (Fig 1) support this hypothesis.

Although this seems to be a general phenomenon, there were some differences between liver and blood DNA, between total and double strand breaks and between the laboratory and field studies. One explanation for the differences between liver and blood may be due to the different physiologies of these two organs (see Part 4 for a discussion). In the present study, the relationship between genotype and strand breakage seems to be stronger for blood than for liver DNA. Further investigation needs to be done to clarify these findings.

Differences between total and double strand breaks may be due to the differing origins or affects of double- and single-

strand breaks (see chapter 3 for a discussion). Double strand breaks are more serious than single-strand breaks in terms of cell lethality, mutagenesis, and DNA repair processes (Kraft et al. 1989, Ward 1988, Kampf and Eicchorn 1983).

Explanations for the differences between the field and the laboratory study include several possibilities. One is that the multitude of biotic and abiotic variables which are present in the field are not present in the laboratory. This may explain why the relationship between genotype and MML was stronger in the laboratory than in the field. In general, the variation in MML was less in the laboratory than it was in the field. Furthermore, in the field the fish received a chronic dose of $\alpha$-, ß- and $\gamma$-radiation. In the laboratory, the fish received an acute dose of X-rays. It has been determined that the type of DNA damage is related to the type of radiation energy absorbed (Kampf and Eichhorn 1983). It is also known that the genetic effects of radiation on fish are more pronounced for acute than chronic radiation (NCRP 1991).

A final explanation for differences in laboratory and field fish could be related to the fact that the fish in Pond 3513 have resided there since 1977, while the laboratory fish have never been exposed to ionizing radiation. Thus, Pond 3513 fish may differ from the laboratory fish due to acclimation or selection.

In most cases, though, similar patterns were seen regardless of the type of strand breakage or tissue examined.

However, some of these patterns were contradictory to what would have been predicted on the basis of genotype frequencies. For example, bands $UBC4_{2055}$ and $UBC4_{1610}$ were more common in contaminated than in reference sites (chapter 4), but individuals with these bands had lower fecundity (chapter 4) and more strand breakage than those without (Table 2). Thus, an increase in frequency of these bands seems to be due to induction rather than selection. It could be that the appearance of these bands signals a deleterious mutation or chromosomal lesion. Alternatively, it could be due to a maladaptive transpositional insertion.

Other molecular mechanisms underlying the relationship between RAPD banding patterns and strand breaks are also unknown, but there are several possibilities. Several of these possibilities lie in the fact that RAPD amplification sites are inverted repeats. Because sites of transposition or chromosomal inversions are typically inverted repeats, it is possible that some RAPDs are amplified from sites of chromosomal rearrangement. For example, it has been found that transposition activity takes place during genotoxic stress. For example, exposure to ionizing radiation leads to an increase in the rearrangements of transposable elements in maize (Doring and Starlinger, 1986) and there have been studies linking DNA repair enzymes, DNA damage, and transposition (Kang et al. 1992). Thus there may be a

relationship between number of RAPD sites, chromosomal rearrangement, and resistance to DNA damage.

Inverted repeats are also present in other DNA structures which have been found to be involved in genotoxic response or resistance. Such structures include: 1) Sites of DNA-nuclear matrix attachment (matrix attachment regions or MARs; Schwartz and Vaughn 1993). The secondary structure of the DNA at these sites is composed of open loops or cruciform structures (Bode et al. 1992, Kowi-Shigematsu and Kowi 1990), which contain inverted repeat sequences (Lewin 1993). 2) Promoter or enhancer elements of DNA repair genes (Kedar et al. 1991). Such regulatory elements are often sites of protein binding, which are themselves often inverted repeats (Lewin 1993). 3) Repetitive DNA (Lewin 1993). Losses or gains in DNA from stressed organisms may occur via repetitive sequences (Cullis 1990, Cullis 1983) and changes in nuclear DNA content have been found as a response to genotoxic stress (Bickham et al. 1992, Theodorakis et al. 1992, Bickham et al. 1998). 4) Amplified genes (Aladjem and Lavi 1992, Quellette et al. 1991). Gene amplification may also contribute to changes in DNA content. Furthermore, such amplification has also been found in cells exposed to carcinogenic (usually genotoxic) chemicals (Berko-Flint et al. 1990, Aladjem et al. 1988, Schimke 1984).

A final possibility is that RAPD amplification sites are located within functional genes or are linked to genes or

other DNA sequences (eg. MARs) which confer a greater ability for DNA repair or resistance to damage. In plants, RAPD markers have been found which are linked to functional genes (Chaparro et al. 1993) and phenotypic traits (Michlemore et al. 1991).

There is always the possibility that the patterns seen here are due to random fluctuations, and not to any adaptive response. For example, it has been suggested that because the number of correlations between number of bands and MML at the 0.05 level of significance is low, this pattern is not much different than would be produced by random chance (G.F. McCracken, University of Tennessee, pers. comm.). However there are three lines of evidence which would tend to refute this hypothesis. First, if we look at the correlations in which the significance level is between 0.10 and 0.05, there are more significant correlations than would be expected by random chance alone. For example, for the field data there are 24 correlations (6 liver and 6 blood, both total and double strand breaks). Thus the expected number of correlations that would be significant at 0.10 and would occur by chance alone is 2.4, but the observed number is 9. For the laboratory data, there are 12 correlations. Thus the expected number of differences significant at 0.10 and due solely to chance is 1.2, but the observed number is 7. Secondly, the correlations between MML and number of bands in the laboratory was highly significant in most cases. Third, there are

several examples in which relationships between RAPD banding patterns and strand breaks have been found for both liver and blood DNA. The similar pattern in both tissues argues against this pattern being due to random chance.

**Allozyme Analysis**

It was found that for liver both genotypes containing the fast allele (FF and FS) had a higher MML than SS individuals, and in blood the order of increasing MML was FS > FF > SS. These results are in accordance with the findings that the frequency of the FS genotype is increased and the frequency of the SS genotype is decreased in contaminated relative to reference sites (Part 5). This provides further evidence in support of the hypothesis that genotype may influence genotoxic response. For comparison purposes, the results from Parts 5 and 6 from the laboratory and from the field are summarized in Table 5.

In considering the role of nucleoside phosphorylase in strand breakage, it is interesting to note that this enzyme is involved in nucleotide synthesis, and that efficient DNA repair is heavily dependant upon the level of nulceotides in the cell (Kunz and Kohlam 1991). In addition, it has been found that one of the responses to X-ray exposure is increase in nucleotide levels (Eckstein et al. 1974). This provides a possible mechanisms whereby NP genotype could be linked with

a physiological adaptation to environmental stress (i.e, repair of radiation-induced DNA lesions) in the contaminated sites. In light of this, perhaps the mechanism behind the NP-strand break relationship lies with the possibility that different NP genotypes may have different kinetic rates. In any case, the relationship between NP genotype, DNA repair and selection in contaminated environments needs further study.

**Conclusions**

This study provides further evidence which suggests that RAPD and allozyme genotypes are capable of detecting changes in population genetic structure as a result of genotoxicant exposure. In particular, it has been shown that in many cases differences in number of bands and frequency of specific bands between contaminated populations and their relationship to fecundity (as seen in Part 5) are in accordance with differences in stand breakage among genotypes. The results of this study along with the finding that strand breakage is correlated to fecundity (Part 4) provide one possible mechanism by which genotoxicant exposure is able to influence population genetic structure. The results from this study may also be applicable to human health. For example, if there is relationship between genotype and DNA-damage resistance or repair in humans, perhaps individuals could be identified

which are at a higher risk of getting cancer or other DNA-damage related diseases.

The hypotheses presented in this discussion provide ample opportunity for exploring the possibilities for the as of yet ambiguous mechanisms linking RAPD and allozyme genotypes to DNA strand breakage. Directions for further study include: 1) Use of molecular biology techniques to examine the relationship between DNA strand breaks and RAPD genotype, 2) Examination of the relationship between other genetic markers and biomarkers of contaminant stress, 3) examination into the genetics and kinetics of nucleoside phosphorylase activities in relation to genotoxicant exposure and DNA damage and repair, 4) determination if there is any relationship between transposable elements or other inverted repeats and DNA repair. Further discussion as to the relationship between genotoxic exposure, DNA strand breakage and genotype and its applicability to the fields of ecotoxicology, environmental biomonitoring and ecological risk assessment can be found in the next section.

**ACKNOWLEDGEMENTS**

The authors would gratefully like to acknowledge W. Connover for his help with the X-ray exposures. We would also like to thank G.F. McCracken and B.P. Bradley for critical review of this manuscript. This project was sponsored in part by the Oak Ridge Y-12 Plant, Department of Environmental Management, Health, Safety, Environment and Accountability Division and by an appointment to the U.S. Department of Energy Laboratory Cooperative Postgraduate Research Training Program administered by the Oak ridge Associated Universities. This project was also supported in part by the U.S. Army Biomedical Research and Development Laboratory (DOE project No. 1061-BO47-A1). The Oak Ridge National Laboratory is administered by Martin Marietta Energy Systems Inc. under contract DE-AC05-A4OR21400 with the U.S. Department of Energy.

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## APPENDIX

Table 1 - Correlation coefficients and significance (P) values for correlations of number of RAPD bands vs. MML for mosquitofish living in a radionuclide-contaminated pond (Pond 3513).

| | | Liver | | Blood | |
|---|---|---|---|---|---|
| Primer* | Strand Break | Corr. Coeff. | P** | Corr. Coeff. | P** |
| OPD2 | Total | 0.000 | >0.50 | 0.000 | >0.50 |
| | Double | -0.08 | 0.48 | 0.080 | 0.35 |
| OPD7 | Total | 0.200 | 0.15 | 0.147 | 0.18 |
| | Double | 0.208 | 0.16 | 0.238 | 0.07 |
| OPD20 | Total | 0.333 | 0.06 | 0.130 | 0.30 |
| | Double | 0.090 | 0.35 | 0.330 | 0.08 |
| UBC4 | Total | -.243 | 0.06 | -0.050 | 0.40 |
| | Double | -.163 | 0.16 | 0.184 | 0.16 |
| UBC9 | Total | 0.467 | 0.01 | 0.176 | 0.17 |
| | Double | 0.257 | 0.05 | 0.100 | 0.31 |

Table 1 (cont')

| Primer* | Strand Break | Corr. Coeff. | P** | Corr. Coeff. | P** |
|---|---|---|---|---|---|
| UBC12 | Total | 0.275 | 0.08 | 0.464 | <0.01 |
| | Double | -0.06 | >0.50 | 0.441 | <0.01 |

*For primers listed here, the number of bands contaminated sites was greater than in reference sites, and a positive correlation of fecundity with number of bands in Pond 3513.

**Probability values for the Kendall correlation test.

Table 2 - Median molecular length (MML) of DNA from mosquitofish with and without particular RAPD bands. Fish were collected from a radionuclide-contaminated pond (Pond 3513). Values represent medians (third quartile/first quartile)*. Numbers in brackets (eg [17]) refer to sample sizes. For each band, sample sizes are the same for total and double strand breaks in the liver and blood. MML values are inversely proportional to the number of strand breaks.

| | | Liver DNA MML | | | Blood DNA MML | | |
|---|---|---|---|---|---|---|---|
| Band** | Strand Break | With Band | Without Band | P† | With Band | Without Band | P |
| $OPD7_{1721}$‡ | Total | 25.5 $(^{+1.7}_{-5.5})$ [17] | 22.3 $(^{+1.5}_{-4.6})$ [11] | 0.20 | 26.5 $(^{+1.1}_{-1.0})$ | 25.0 $(^{+2.0}_{-8.6})$ | 0.05 |
| | Double | 27.0 $(^{+3.4}_{-4.0})$ | 27.6 $(^{+1.3}_{-2.8})$ | 0.25 | 26.8 $(^{+0.1}_{-1.3})$ | 26.5 $(^{+0.3}_{-0.8})$ | 0.23 |
| $OPD7_{1390}$‡ | Total | 23.6 $(^{+1.7}_{-2.7})$ [19] | 22.5 $(^{+3.5}_{-5.3})$ [9] | 0.15 | 26.7 $(^{+1.8}_{-1.5})$ | 25.0 $(^{+2.0}_{-9.0})$ | 0.03 |

Table 2 (cont')

| Band* | Strand Break | With Band | Without Band | P** | With Band | Without Band | P |
|---|---|---|---|---|---|---|---|
| | Double | 28.0 $(^{+1.7}_{-1.6})$ | 27.0 $(^{+0.0}_{-1.9})$ | 0.07 | 27.0 $(^{+1.5}_{-0.6})$ | 26.4 $(^{+0.6}_{-3.4})$ | 0.13 |
| $OPD13_{677}$§ | Total | 25.4 $(^{+0.1}_{-0.1})$ [4] | 21.6 $(^{+1.4}_{-5.5})$ [24] | 0.03 | 25.1 $(^{+6.0}_{-0.1})$ | 26.5 $(^{+1.7}_{-5.7})$ | 0.27 |
| | Double | 27.8 $(^{+2.7}_{-2.7})$ | 27.0 $(^{+2.0}_{-5.8})$ | 0.27 | 27.0 $(^{+0.4}_{-0.3})$ | 26.5 $(^{+0.3}_{-0.8})$ | 0.23 |
| $OPD13_{595}$ | Total | 23.0 $(^{+1.5}_{-4.5})$ [8] | 22.2 $(^{+2.4}_{-4.4})$ [20] | 0.26 | 26.4 $(^{+0.8}_{-0.6})$ | 25.0 $(^{+1.1}_{-6.2})$ | 0.11 |
| | Double | 28.5 $(^{+1.4}_{-5.3})$ | 26.0 $(^{+1.3}_{-1.7})$ | 0.36 | 26.9 $(^{+0.4}_{-0.6})$ | 26.0 $(^{+0.3}_{-3.5})$ | 0.19 |

Table 2 (cont')

| Band* | Strand Break | With Band | Without Band | P** | With Band | Without Band | P |
|---|---|---|---|---|---|---|---|
| $OPD20_{433}$‡ | Total | 24.0 ($^{+7.0}_{-2.0}$) [17] | 23.0 ($^{+1.2}_{-8.6}$) [11] | 0.50 | 26.8 ($^{+2.5}_{-4.8}$) | 24.5 ($^{+2.0}_{-11.7}$) | 0.05 |
| | Double | 24.5 ($^{+7.8}_{-3.0}$) | 27.0 ($^{+3.0}_{-2.0}$) | 0.17 | 27.0 ($^{+1.0}_{-2.0}$) | 24.7 ($^{+2.6}_{-3.0}$) | 0.02 |
| | Double | 27.6 ($^{+1.7}_{-2.6}$) | 29.0 ($^{+0.6}_{-2.0}$) | 0.50 | 26.0 ($^{+1.4}_{-1.3}$) | 26.4 ($^{+0.4}_{-0.2}$) | 0.30 |
| $UBC4_{1407}$‡ | Total | 22.9 ($^{+0.9}_{-5.4}$) [11] | 24.0 ($^{+0.9}_{-3.7}$) [17] | 0.10 | 27.8 ($^{+1.3}_{-1.0}$) | 25.1 ($^{+1.8}_{-8.6}$) | 0.03 |
| | Double | 27.7 ($^{+3.2}_{-8.7}$) | 27.6 ($^{+2.8}_{-2.5}$) | 0.50 | 26.3 ($^{+1.6}_{-0.6}$) | 26.0 ($^{+1.0}_{-3.0}$) | 0.43 |
| $UBC4_{440}$ | Total | 22.8 ($^{+2.5}_{-3.2}$) [24] | 23.4 ($^{+1.2}_{-5.6}$) [4] | 0.50 | 26.2 ($^{+1.0}_{-1.1}$) | 22.8 ($^{+3.9}_{-6.5}$) | 0.33 |
| | Double | 27.6 ($^{+2.8}_{-2.8}$) | 29.3 ($^{+3.5}_{-1.6}$) | 0.30 | 26.4 ($^{+0.3}_{-0.9}$) | 25.5 ($^{+1.1}_{-4.2}$) | 0.23 |

Table 2 (cont')

| Band* | Strand Break | With Band | Without Band | P** | With Band | Without Band | P |
|---|---|---|---|---|---|---|---|
| $UBC6_{575}$‡ | Total | 23.9 $(^{+1.5}_{-3.1})$ [18] | 18.3 $(^{+3.8}_{-3.4})$ [10] | 0.04 | 27.0 $(^{+1.3}_{-1.3})$ | 25.0 $(^{+1.0}_{-8.5})$ | 0.01 |
| | Double | 29.3 $(^{+1.0}_{-1.7})$ | 24.5 $(^{+0.8}_{-4.8})$ | 0.02 | 26.6 $(^{+0.6}_{-1.0})$ | 24.9 $(^{+1.9}_{-1.4})$ | 0.04 |
| $UBC6_{455}$‡ | Total | 22.4 $(^{+1.6}_{-3.4})$ [11] | 23.4 $(^{+1.3}_{-1.5})$ [17] | 0.40 | 27.2 $(^{+1.8}_{-1.0})$ | 25.0 $(^{+0.9}_{-3.6})$ | 0.01 |
| | Double | 27.2 $(^{+2.4}_{-2.7})$ | 26.3 $(^{+2.5}_{-1.6})$ | 0.09 | 27.0 $(^{+7.6}_{-1.0})$ | 25.6 $(^{+1.8}_{-1.3})$ | 0.07 |
| $UBC9_{921}$ | Total | 23.5 $(^{+2.0}_{-7.0})$ [21] | 18.3 $(^{+6.5}_{-10.3})$ [7] | 0.15 | 26.5 $(^{+1.1}_{-1.5})$ | 27.2 $(^{+1.0}_{-1.2})$ | 0.40 |
| | Double | 27.6 $(^{+1.3}_{-3.1})$ | 25.6 $(^{+4.4}_{-9.0})$ | 0.20 | 26.3 $(^{+0.6}_{-1.2})$ | 26.4 $(^{+0.8}_{-1.0})$ | 0.45 |

Table 2 (cont')

| Band* | Strand Break | With Band | Without Band | P** | With Band | Without Band | P |
|---|---|---|---|---|---|---|---|
| $UBC12_{1269}$‡ | Total | 23.9 $(^{+1.0}_{-2.2})$ [17] | 20.8 $(^{+4.0}_{-3.6})$ [11] | 0.10 | 26.2 $(^{+1.1}_{-1.2})$ | 21.7 $(^{+3.4}_{-6.4})$ | 0.02 |
| | Double | 27.4 $(^{+1.5}_{-2.3})$ | 25.1 $(^{+4.4}_{-2.1})$ | 0.20 | 27.0 $(^{+1.0}_{-1.0})$ | 24.7 $(^{+1/3}_{-1.4})$ | 0.01 |
| $UBC12_{1158}$‡ | Total | 25.0 $(^{+0.4}_{-1.6})$ [5] | 19.6 $(^{+1.2}_{-4.8})$ [23] | 0.04 | 27.0 $(^{+3.0}_{-2.0})$ | 25.4 $(^{+0.6}_{-8.9})$ | 0.06 |
| | Double | 27.4 $(^{+1.7}_{-2.4})$ | 25.1 $(^{+1.9}_{-4.5})$ | 0.06 | 26.4 $(^{+0.6}_{-0.4})$ | 24.7 $(^{+0.0}_{-6.4})$ | 0.01 |
| $UBC16_{1016}$‡ | Total | 23.8 $(^{+1.6}_{-5.0})$ [19] | 20.8 $(^{+2.4}_{-5.5})$ [9] | 0.06 | 25.8 $(^{+1.0}_{-0.8})$ | 24.0 $(^{+2.0}_{-2.0})$ | 0.01 |
| | Double | 28.5 $(^{+1.9}_{-1.6})$ | 24.0 $(^{+1.0}_{-3.4})$ | 0.01 | 26.5 $(^{+0.2}_{-0.6})$ | 24.8 $(^{+1.1}_{-1.1})$ | 0.01 |

[a]Quartiles are a non-parametric measure of variance roughly analogous to standard deviation. I.e., 1/4 of the population have values less than the first quartile and 1/4 have values which are greater than the third quartile.

Table 2 (cont')

**The bands listed above were chosen for analysis because they were present at a higher frequency in contaminated than non-contaminated populations (see Part 5).

†Probability values for the test of $H_o$: No difference between individuals with and without each band; Wilcoxon Rank-Sum test.

‡Primers for which $P \leq 0.05$ in at least one comparison.



§Primers for which $P \leq 0.10$ in at least one comparison.

Table 3 - Correlation coefficients and significance (P) values for correlations of number of RAPD bands vs. MML for mosquitofish exposed to X-rays in the laboratory. Primers were included in this table only if they showed at least one significant correlation

| Primer | Strand Break | Corr. Coeff. | P - value |
|---|---|---|---|
| OPD7* | Total | 0.481 | <0.01 |
| | Double | 0.457 | <0.01 |
| OPD20* | Total | 0.170 | 0.10 |
| | Double | 0.07 | 0.31 |
| UBC6** | Total | 0.200 | 0.06 |
| | Double | 0.00 | 0.50 |
| UBC9* | Total | 0.288 | 0.02 |
| | Double | 0.235 | 0.04 |
| UBC12** | Total | 0.484 | <0.01 |
| | Double | 0.299 | 0.01 |

*For these primers there was a difference in average number of bands between contaminated and non-contaminated sites, and a positive

Table 3 (cont')

correlation between number of RAPD bands and fecundity. (see Part 5).

** For these primers there was a positive correlation between fecundity and number of bands (see Part 5).

Table 4 - Median molecular length (MML) of liver DNA from mosquitofish with and without particular RAPD bands. Fish were exposed to X-rays in the laboratory. Values represent medians (+third quartile/-first quartile)*. Numbers in brackets (eg., [17]) refer to sample sizes. For each band, sample sizes are the same for total and double strand breaks. MML values are inversely proportional to the number of strand breaks.

| Band | Strand Break | With Band | Without Band | P |
|---|---|---|---|---|
| $OPD7_{1721}$ | Total | 13.5 $(^{+1.4}_{-1.9})$ [14] | 11.2 $(^{+2.9}_{-5.9})$ [14] | 0.05 |
| | Double | 22.0 $(^{+1.1}_{-0.9})$ | 21.0 $(^{+1.2}_{-2.2})$ | 0.10 |
| $OPD20_{605}$ | Total | 13.5 $(^{+2.2}_{-6.8})$ [18] | 11.8 $(^{+1.6}_{-2.1})$ [10] | 0.15 |
| | Double | 21.9 $(^{+1.0}_{-2.1})$ | 21.7 $(^{+0.3}_{-1.7})$ | 0.39 |
| $OPD20_{433}$ | Total | 13.5 $(^{+1.1}_{-7.0})$ [20] | 11.8 $(^{+2.3}_{-0.3})$ [8] | 0.15 |
| | Double | 21.9 $(^{+1.0}_{-2.1})$ | 21.7 $(^{+0.3}_{-0.7})$ | 0.39 |
| $UBC2_{908}$ | Total | 15.0 $(^{+1.0}_{-2.3})$ [10] | 11.8 $(^{+1.8}_{-5.9})$ [18] | 0.02 |
| | Double | 22.1 $(^{+1.8}_{-1.3})$ | 21.7 $(^{+0.8}_{-1.5})$ | 0.28 |
| $UBC4_{2055}$ | Total | 12.9 $(^{+0.5}_{-3.4})$ [16] | 13.9 $(^{+0.8}_{-7.3})$ [12] | 0.23 |
| | Double | 22.0 $(^{+0.5}_{-1.3})$ | 21.8 $(^{+0.5}_{-2.1})$ | 0.32 |

Table 4 (cont')

| Band | Strand Break | With Band | Without Band | P |
|---|---|---|---|---|
| $UBC4_{1212}$ | Total | 13.4 $(^{+1.8}_{-3.4})$ [24] | 6.0 $(^{+6.5}_{-2.8})$ [4] | 0.10 |
| | Double | 21.9 $(^{+0.5}_{-0.7})$ | 19.8 $(^{+2.7}_{-0.6})$ | 0.19 |
| $UBC6_{575}$ | Total | 13.4 $(^{+1.7}_{-4.1})$ [15] | 12.9 $(^{+1.3}_{-5.1})$ [13] | 0.50 |
| | Double | 21.8 $(^{+0.8}_{-2.3})$ | 21.8 $(^{+0.9}_{-1.5})$ | 0.50 |
| $UBC9_{921}$ | Total | 13.5 $(^{+2.2}_{-3.2})$ [19] | 9.3 $(^{+4.0}_{-3.7})$ [9] | 0.03 |
| | Double | 22.1 $(^{+1.5}_{-0.9})$ | 21.7 $(^{+0.5}_{-2.0})$ | 0.20 |
| $UBC12_{1269}$ | Total | 14.7 $(^{+2.7}_{-4.4})$ [11] | 12.7 $(^{+0.5}_{-6.4})$ [17] | 0.02 |
| | Double | 22.0 $(^{+0.8}_{-0.8})$ | 21.7 $(^{+0.6}_{-1.6})$ | 0.26 |
| $UBC16_{1016}$ | Total | 13.4 $(^{+1.7}_{-2.4})$ [14] | 11.5 $(^{+2.3}_{-5.1})$ [14] | 0.03 |
| | Double | 22.2 $(^{+1.0}_{-0.5})$ | 21.1 $(^{+0.8}_{-1.8})$ | 0.20 |

*Quartiles are a non-parametric measure of variance roughly analogous to standard deviation. I.e., 1/4 of the population have values less than the first quartile and 1/4 have values which are greater than the third quartile.

Table 5 - Qualitative summary of the data examining 1) differences in RAPD genotype (in terms of genetic diversity, average number of bands, or frequency of individual bands) between contaminated and non-contaminated populations 2) RAPD genotype-specific differences in fecudnity and DNA damage between individuals within a radionuclide-contaminated population (Pond 3513).

| | | Number of Bands | | | | Individual Bands | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Correlation† | | | With vs. Without§ | | | |
| Primer | Div* | Popn. Diffs** | Fecdy | Strand Breaks Fld | Strand Breaks Lab | Popn Freq Diffs‡ | Fecdy | Strand Breaks Fld | Strand Breaks Lab |
| OPD2 | - | + | = | = | = | + 1586, 1060 | ND* | ND | ND |
| OPD7 | + | + | + | + | + | + 1721, 1390 | + 1390 | - 1721, 1390 | - 1721 |

Table 5 (cont')

| | | Number of Bands | | | | Individual Bands | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Correlation† | | | | With vs. Without§ | | |
| Primer | Div* | Popn. Diffs** | Fecdy | Strand Breaks Fld | Lab | Popn Freq Diffs‡ | Fecdy | Strand Breaks Fld | Lab |
| OPD8 | + | - | + | = | = | - 560 | = | = | ND |
| OPD13 | + | - | + | = | = | + 428<br>- 677,<br>595,<br>263 | = | = | ND |
| OPD18 | + | = | = | = | = | = | = | = | ND |
| OPD20 | + | + | = | - | - | + 605,<br>433 | + 605,<br>433 | - 433 | = |

Table 5 (cont')

| | Number of Bands | | | | Individual Bands | | | |
|---|---|---|---|---|---|---|---|---|
| | | Correlation† | | | With vs. Without§ | | | |
| Primer | Div* | Popn. Diffs** | Fecdy | Strand Breaks Fld | Lab | Popn Freq Diffs‡ | Fecdy | Strand Breaks Fld | Lab |
| UBC1 | = | = | = | = | = | = | ND | ND | ND |
| UBC2 | = | = | = | = | = | = | ND | ND | ND |
| UBC4 | - | + | = | = | = | + 2055, 1610, 1407 | - 1610 | - 1610 | - 1610 |
| UBC6 | + | = | + | - | - | + 575, 455 | + 575, 455 | - 575, 455 | = |

Table 5 (cont')

| | | Number of Bands | | | | Individual Bands | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Correlation† | | | With vs. Without§ | | | |
| Primer | Div* | Popn. Diffs** | Fecdy | Strand Breaks Fld | Lab | Popn Freq Diffs‡ | Fecdy | Strand Breaks Fld | Lab |
| UBC9 | + | + | + | - | - | + 699 | + 921 | = | - 921 |
| UBC16 | + | = | = | = | = | + 1016 | + 1016 | - 1100, 1016 | - 1100 |

*Denotes differences in genetic diversity between contaminated and control sites. "+" indicates that the diversity was higher in contaminated than in reference sites, "-" indicates the reverse, and "=" indicates no difference

**Denotes differences in average number of bands between contaminated and reference sites. "+", "-", and "=" are as described above

Table 5 (cont')

†Signifies correlations between number of bands and fecundity or amount of DNA strand breakage for fish residing in Pond 3513. "+", "-", and "=" denote positive, negative, and no correlations, respectively. "Fld" indicates a correlation determined in the field (Pond 3513), "Lab" indicates results from X-ray exposure in the laboratory.

‡Denotes differences between contaminated and control populations in terms of frequency of the bands produced by the indicated primers. "+", "-", and "=" indicate that the frequency of such bands in contaminated sites is higher, lower, or not different from reference sites, respectively. The numbers listed indicate the molecular length of the bands showing such differences. For example, for primer OPD20, "+ 605, 433" indicates that bands $OPD20_{605}$ and $OPD20_{433}$ were present at a higher frequency in the contaminated than reference sites.

§Indicates differences in fecundity (Fecdy) or DNA strand breaks between fish in Pond 3513 with and without specific bands produced by each primer. In terms of fecundity, "+", "-", and "=" indicate that fish in Pond 3513 withh these bands had a higher

Table 5 (cont')

fecundity than did fish without them. In terms of strand breaks "+", "-", and "=" indicate that fish with these bands had more, less or the same amount of DNA strand breakage than did fish without these bands. For example, for primer OPD20, "+ 605, 433" indicates that fish in Pond 3513 with these bands $OPD20_{605}$ and $OPD20_{433}$ had fewer strand breaks than did fish without these bands. Fld = data from a field study (Pond 3513), Lab indicates data from a laboratory study. For example, for primer OPD20, under the heading "Strand Breaks", "+ 605, 433" indicates that fish in Pond 3513 with these bands $OPD20_{605}$ and $OPD20_{433}$ had a higher MML (i.e., fewer strand breaks) than did fish without these bands.

**Figure Legends**

Figure 1 - Correlation between frequency of certain RAPD bands and median molecular length (MML) difference for each band (MML of fish with that band - MML of fish without that band). Only bands which had a higher frequency in contaminated than non-contaminated ponds were used. Data are for fish from Pond 3513.

Figure 2 - Median molecular length (MML) for fish with different genotypes at the nucleoside phosphorylase (NP) locus. F = fast allele, S = slow allele, FF and SS are homozygotes, FS is a heterozygote. Bars represent medians, error bars represent first and third quartiles. Bars labeled with the same letter are not significantly different at the 0.05 level (Kruskall-Wallace test).

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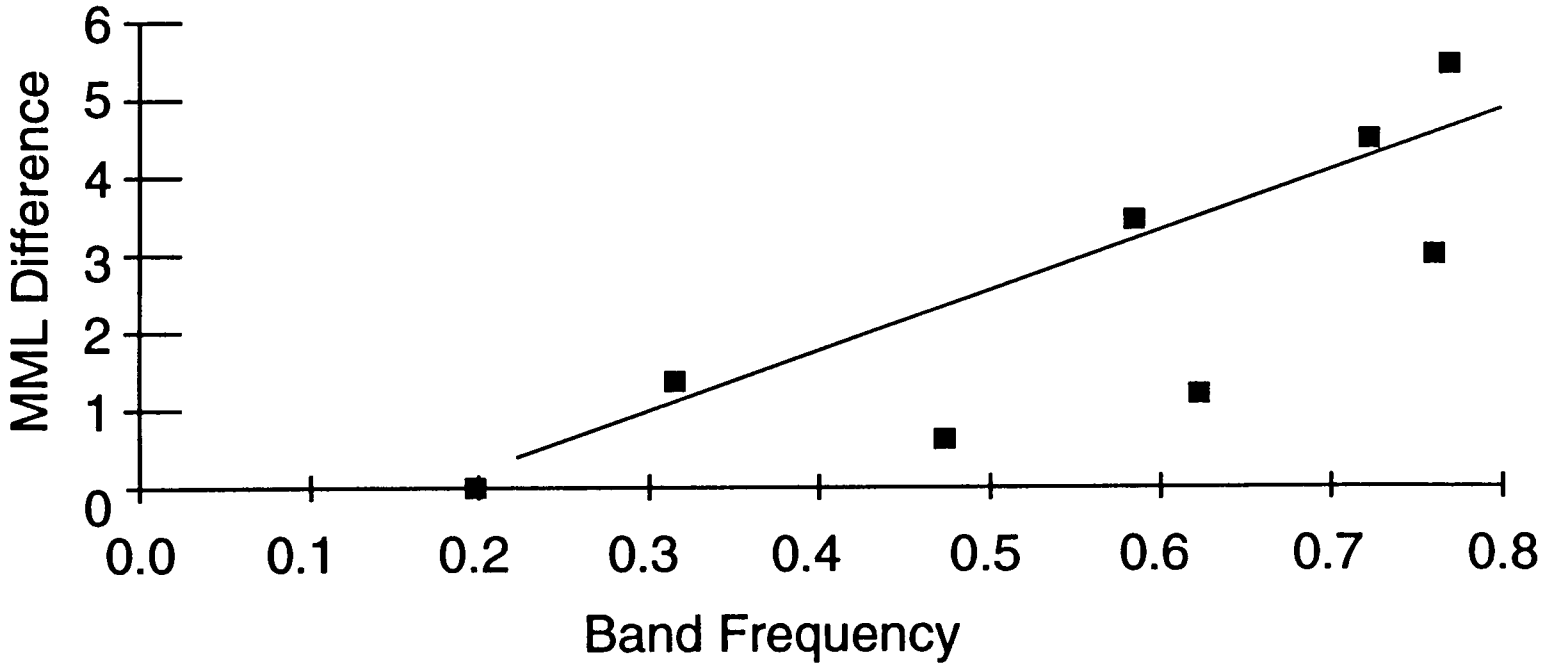

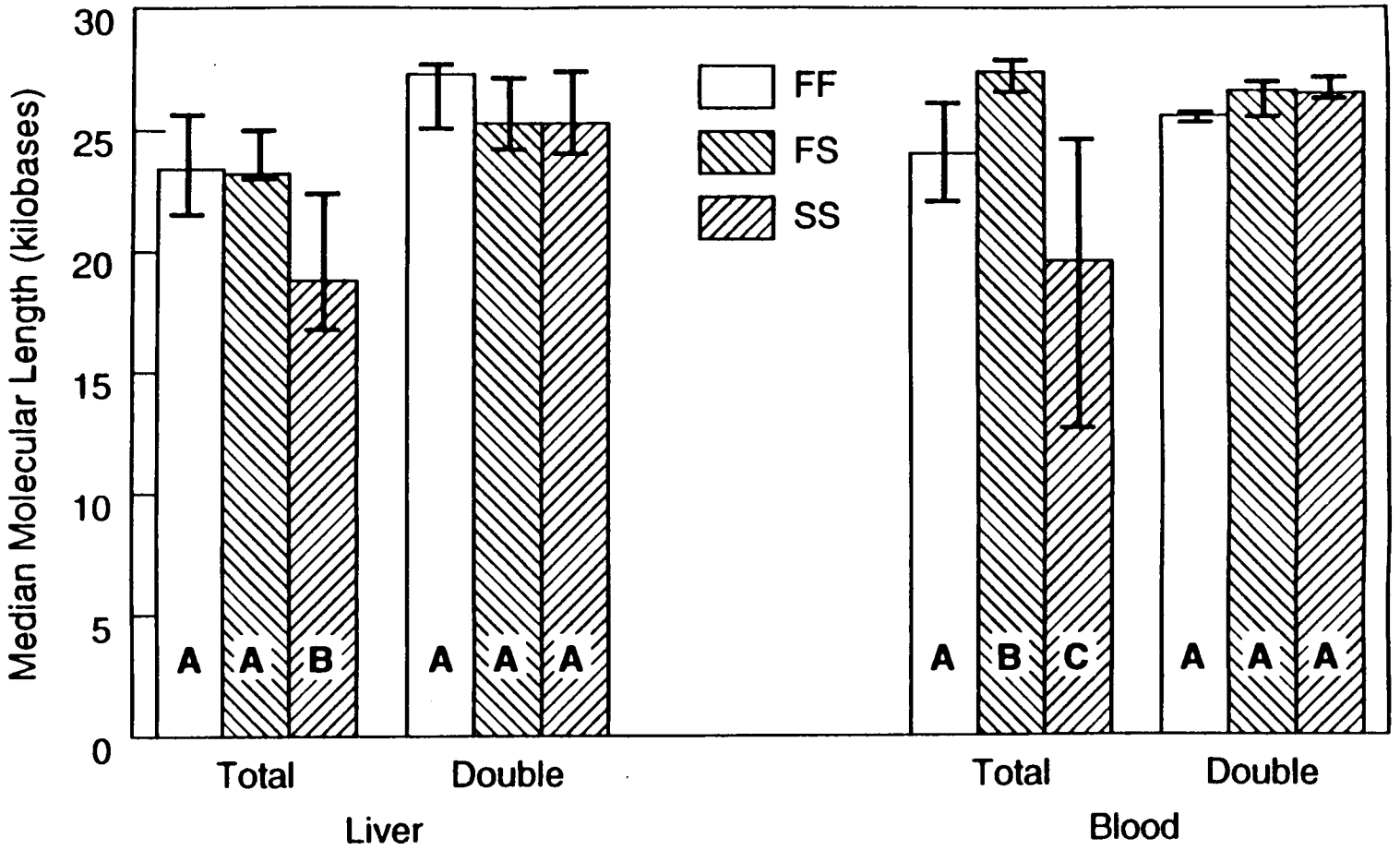
Median Molecular Length (kilobases)
30
25
20
15
10
5
0
FF
FS
SS
A
A
B
A
A
A
A
B
C
A
A
A
Total
Double
Liver
Total
Double
Blood

# PART 7

# DISCUSSION: RELEVANCE TO ENVIRONMENTAL MONITORING AND RISK ASSESSMENT

## INTRODUCTION

The results of this dissertation have shown that genotoxic exposure can influence the physiology and population genetic structure in both bluegill sunfish and mosquitofish. The ultimate objective of any study in biomarkers is that the information gained will be used for environmental biomonitoring or ecological risk assessment. Biomarker-based biomonitoring involves the use of anatomical, biochemical, or molecular parameters in order to demonstrate that organisms have been exposed to environmental contaminants and that "toxicants have entered organisms, been distributed within the tissues, and are eliciting a toxicological effect at critical targets." (McCarthy and Shugart 1990, pg. 6). Ecological risk assessment involves hazard identification, exposure assessment and effects assessment. Hazard identification involves elucidation of the environmental parameters at risk and methods by which to measure them; a spatial and temporal description of the environment under consideration; and a description of the amounts and frequencies of chemical releases. Exposure and effects assessments determine how much contaminant reaches the organisms and its impacts on homeostasis of the organism. These data are used to "estimate the magnitude and likelihood of (ecological) effects" (Suter 1990). Thus this chapter will relate the results of this dissertation with regards to the above discussion.

Chapters 1 and 2 dealt primarily with expression of biomarkers in sunfish exposed to contaminated sediment. The last three chapters dealt with the ecotoxicology of radionuclides in mosquitofish. Therefore these two topics will be discussed separately in relation to environmental biomonitoring, ecological risk assessment and directions for future research.

The major contribution of these chapters to ecological risk assessment or biomonitoring are that they dealt with multiple biomarkers, they related field and laboratory studies, and that they examine novel approaches to studying biomarkers.

The study of suites of biomarkers such as was done in Part 2 obfuscates significant for a number of reasons. First, comparative studies offer insights into the mechanisms of sublethal toxicity that single-endpoint studies cannot. Secondly, organisms in the field are most often exposed to complex mixtures of organic and inorganic contaminants. In order to monitor for effects of many different toxins, one must examine a wide variety of response that may be indicative of specific contaminants. Furthermore, although genotoxic compounds can cause DNA damage, this is not their only effect. For example, somatic effects of PAHs include membrane enzyme inhibition (Danga and Masurekar 1989), immune dysfunction (Weeks et al. 1990), and reduction of hematocrit (Cassillas et al. 1985). A clearer understanding of the underlying principles governing genotoxic response may be obtained by monitoring somatic as well as genetic responses to genotoxic compounds.

Monitoring multiple biomarkers also has implications for ecological risk assessment. It has been suggested that the

assignment of a real ecological affect to environmental contamination is facilitated if a number of ecological variables (n) are measured to construct an n-dimensional "ecological state space" (Suter 1990). This can be used to estimate the probability that the state space of a contaminated population lies outside that of the reference sites. Because most biomarkers do not have a clearly defined toxicological threshold, the state-space approach may be more appropriate when using biomarkers to assign cause and effect relationships (Suter 1990). The use of multivariate statistics may be useful in constructing such a space, and can include such analyses as canonical or discriminant analysis (eg. see Adams et al. 1990). Additionally, Suter (1990) has stated that in order for biomarkers to be incorporated into risk assessments they must "increase in a regular and predictable manner with increasing exposure" (Suter 1990). This is more likely to be true for a suite of biomarkers than it is for individual biomarkers. Again, multivariate statistics may be useful for this purpose.

Besides examining multiple endpoints of exposure, this study is also significant in that it relates results from the laboratory to those from the field. Two of the components of ecological risk assessment are 1) establishing if the effects of pollution on communities are due to contaminants or to variation in some other ecological parameter and 2) determining the cause (Suter 1990). By verifying field

observations with laboratory exposures and vice versa both of these objectives can be more readily accomplished.

A final contribution of chapters 1 and 2 to the field of biomonitoring is that they dealt with novel approaches for studying biomarkers. There were several novel approaches detailed in these chapters, for both somatic and genetic biomarkers. Two examples of these - zinc protoporphyrins and chromosomal proteins, a somatic and genetic biomarker, respectively - will be summarized and discussed below to illustrate how these novel biomarkers can benefit biomonitoring programs.

Monitoring zinc protoporphyrin (ZPP) levels has been widely used in human toxicology, unlike environmental toxicology. Elevated ZPP levels can be indicative of chronic heavy metal exposure (Cory-Slechta et al. 1989). Although altered zinc protoporphyrins are indicative of exposure, they can also be thought of as a biomarker of effect, because physiological basis of its expression is aberrant iron metabolism or anemia (Labbe and Rettmer 1989). In addition, aberrant porphyrin levels may differentiate between chronic and acute exposures. ZPP is mainly indicative of chronic effects (Labbe and Rettmer 1989), and in chapter 1 it was shown that higher levels are characteristic of longer exposures. Elevated levels of free porphyrin (i.e., heme precursors not chelated with any metal), however, are more indicative of acute heavy metal exposure (Hirsch 1989). More

experiments need to be performed in order to determine if these two types of aberrant porphyrins can distinguish between acute and chronic metal stress in the field. Such information would be a definite boon to ecological biomonitoring and risk assessment.

Another biomarker which was novel to environmental biomonitoring was chromosomal protein profiles. There are several other recent studies which have found that chromatin proteins are involved in genotoxic response (Althaus et al. 1993, Wagner et al. 1993, D'Surney et al. 1993), so this approach appears to be widely applicable. This technique has advantages over other methods of detecting DNA damage. DNA repair processes may obscure results from DNA damage assays; monitoring alterations in chromatin protein profiles could obviate this problem.

A novel approach to studying DNA damage was discussed in chapter 2. In this chapter electrophoresis was employed to examine DNA damage in genotoxicant-exposed fish. Although this method for studying strand breaks has been in use for quite some time in radiobiology and biochemistry, its application in environmental monitoring has yet to be appreciated. This approach has several advantages over other methods of examining DNA strand breakage, and this was discussed in detail in chapter 2. A further aspect of this study was that blood was used to examine DNA strand breakage. Besides the fact that collection of blood can be less labor

intensive than sampling other types of tissues, this type of non-destructive monitoring has other applications. In a social environment which is becoming increasingly critical of animal research, the role of non-destructive biomonitoring will probably play a larger role in biomonitoring in the future. In chapter 3 a related aspect of this study was comparing DNA damage in blood and in liver tissues. In order for blood to confidently be used as a surrogate for other tissues, the relationship between biomarkers in the blood and in other target tissues must be fully understood.

The results from these studies also suggest avenues of future research. These possibilities include: 1) Continued investigation into the novel biomarkers and approaches discussed above, 2) further investigation into the relationship between gentoxicant exposure, body burdens and biomarker expression, 3) further comparisons of multiple biomarkers to determine the relationships between them, 4) determination of the relationship between biomarker expression and higher-level effects both on the individual (eg. behavior, reproduction, bioenergetics, etc.) and on the ecological scale (eg. population and community responses) and 5) additional investigations of the use of blood or other non-destructive biomarkers.

# GENETIC TOXICOLOGY OF RADIONUCLIDES IN MOSQUITOFISH

Genetic ecotoxicology can be defined as the study of the effects of xenobiotic compounds on DNA structure and function and their relationship to higher-order ecological effects. This broad category includes DNA damage and repair, effects of contaminants on gene expression, and population genetics in contaminated environments. Examination of DNA damage and repair can give information about exposure of organisms to genotoxic chemicals. Examination of the effects of contaminants on gene expression may give insights into the identification of contaminants present and determination of their effects. Determination of the effects of contaminants on population genetic structure may provide information about the effects of contaminants at the population level. Therefore the study of the effects of anthropogenic contaminants on DNA structure and function should be an essential component of ecotoxicology, biomonitoring, and ecological risk assessment.

The objective of this paper is to examine the role of genetic ecotoxicology in biomonitoring and ecological risk assessment. In order to do this we will summarize recent results that we have obtained from the study of radionuclide-contaminated mosquitofish populations, and discuss them in relation to environmental biomonitoring and risk assessment. These studies have focused on the effect of radionuclides on

DNA damage and population genetic structure. This will be followed by concluding remarks concerning the role of genetic ecotoxicology in general.

**Radionuclides in Mosquitofish**

Chapters 3-5 have described the effects of radionuclide contamination on mosquitofish populations. In the chapter 3 it was established that populations living in radionuclide-contaminated environments displayed more strand breakage than individuals in non-contaminated environments. Also, within a contaminated environment, the degree of strand breakage was correlated with fecundity. It could very well be that the relationship between strand breakage and fecundity is an indirect one. It may be that both responses are correlated to a third parameter. In terms of biomonitoring or ecological risk assessment, it may not matter much if strand breakage is an effector or co-correlate of fecundity, the result is the same. In either case monitoring of strand breakage may contribute to predictions or assessments of the effects of genotoxic exposure on fecundity of the population. Further research is need to determine if effects on fecundity have impacts on population dynamics and demographics.

The relationship between genotoxic exposure/effects and fecundity is of significance because fecundity is a key factor linking individual responses to population, community or

ecosystem effects. If this pattern is a general phenomenon, effects of genotoxins on fecundity can influence population parameters such as recruitment and population growth as well as population genetic structure. There may also be affects on the community structure as well: if the genotoxicity/fecundity relationship differs between different species, this may affect interspecies competition or predation, and ultimately community structure.

The correlation between fecundity and strand breakage also has implications for the evolution of mosquitofish populations which have an increased resistance to genotoxic effects. Such resistance may have implications for ecological risk assessment. In certain circumstances, individuals which are adapted for a contaminated environment have a disadvantage in a non-contaminated environment (eg., Meharg and Macnair 1990). This could have negative ecological effects if the contaminant stress is removed or if the level of effects or exposure is variable over time (Benjamin 1993). Also, genotypes which confer greater resistance to genotoxic contamination may be more sensitive to other forms of stress. In one such study, Trabalka and Allen (1977) found that mosquitofish from a radioactively-contaminated pond had a lower thermal tolerance than those from reference sites. Direct effects of radiation on thermal tolerance could be ruled out because laboratory-reared descendants of the contaminated population were used in this study.

Chapter 4 examined the population structure in radionuclide-contaminated populations and reference sites using the RAPD and allozyme techniques. Both showed an increase in population diversity in contaminated populations. Possibly, an increase in diversity may be due to an increase in genetic rearrangement or mutation rate. These rearrangements may include chromosomal aberrations. An increased chromosomal aberration and mutation rate may have deleterious ecological effects. For example, mosquitofish in a radionuclide-contaminated reservoir displayed a higher number of abnormal embryos than a reference site (Blaylock 1980). When these fish were reared in the laboratory, subsequent generations also showed an increased frequency of embryonic abnormalities, suggesting that there was an increased genetic load of deleterious mutations in this population (Tabalka and Allen 1977). Since abnormal embryos are not viable, an increased number of abnormalities may lead to reduced reproductive output of the population. If RAPD diversity is related to mutation rate, then monitoring such diversity would be a valuable contribution to determination of adverse affects of genetic insult on populations.

A second finding in this chapter was that certain RAPD banding patterns were more common in the contaminated sites than were others. It was found that both the number of bands per individual and the frequency of certain bands differed between contaminated and non-contaminated populations. Thus

such parameters may serve as population genetic markers to aid in identifying populations which are subject to genotoxin-induced selection. The use of such indicators has advantages over individual-based biomarkers of genotoxic contamination. For example, the use of DNA damage has been used as indicator or genotoxic expose, but if an organism has an efficient DNA repair or other genotoxic compensatory response, the effects in the organism may not be evident. In one instance, Gambusia collected from a radioactively-contaminated pond and analyzed for DNA lesions via flow cytometry were not different from a reference site (J.W. Bickham, U. of Texas, pers. comm.). In these cases perhaps examination of RAPD genotypes may be more indicative of genotoxin exposure.

Finally, chapter 5 examined the relationship between DNA strand breakage and genotype. An interesting outcome of these studies was that fish with RAPD banding patterns that were more common in contaminated environments had a lower level of DNA strand breakage both in the field and when exposed to X-rays in the laboratory. This suggests that such banding patterns may provide a benefit to fish living in contaminated environments in terms of protection or repair of DNA strand breakage, and that this benefit may have an adaptive value. In any case, the relationship between a biomarker (strand breakage) and population genetics is important. There are a multitude of environmental variables besides xenobiotic contamination which could affect population genetic structure.

But biomarkers indicative of contaminant exposure may provide evidence that changes in population genetics are influenced by contaminant exposure. A relationship between biomarker expression and genotype provides further evidence indicating these changes were contaminant-induced. These considerations need to be taken into account to make population genetics more useful in ecological risk assessment.

**Genetic Ecotoxicology: A Review and Discussion**

The discussion above focused on two aspects of genetic ecotoxicology - DNA damage/repair and population genetics - and their possible relationship to ecosystem level responses. These two aspects of genetic ecotoxicology will be discussed and reviewed in the context of current or future implications to environmental risk assessment and biomonitoring.

DNA Damage and Repair - The study of DNA damage and repair is an important aspect of ecotoxicology for two reasons. One reason is that DNA is the fundamental unit of inheritance and reproduction, so that perturbations in its structure and function could lead to changes in population dynamics or demography. For instance, it has been found that radionuclide contamination reduces the average fecundity of some fish populations (reviewed in NCRP 1991). Genetic damage has also been implicated in the aging process (Kirkwood 1989).

Related to this is the finding that exposure to ionizing radiation has a detrimental effect on longevity in laboratory animals (Lindrop and Sacher 1966, Grahn 1968) Also, effects of radiation are more pronounced for older and younger animals than for ones at an intermediate age (Casarett 1968). Thus exposure to genotoxic compounds may have impacts on population age structures. For example, it has been found that bullhead catfish (Ictalarus nebulosus) populations living in PAH-contaminated environments have age-structures which are different from reference sites (P.F. Bauman, Ohio State Univeristy, pers. comm.). Because the size (which in many poikilotherms is directly correlated with age) of an animal has implications for survival, fecundity, bioenergetics and behavior, any perturbation in size structures has the potential to have affects at the population level or above. The sex structure of populations may also be affected, because there may be sex differences in the response to DNA damage (Casarett 1968). Studies examining these relationships would be an integral part of ecotoxicology.

Another reason DNA damage and repair is important to ecotoxicology is that genotoxins are ubiquitous and are released in large quantities into the environment. For example, two very common contaminants which can lead to DNA damage are PAHs (McElroy et al. 1989) and phthalate esters (Yang et al. 1990, Reddy and Lalwai 1982). Environmental sources of these include fossil fuel combustion (McElroy et

al. 1989) and plastics (Graham 1973), two things which are very prevalent in our society.

Population Genetics - Certain aspects of population genetic studies can be useful in studying the effects of environmental contamination. These include determination of genetic variation within and between populations, genotype frequencies, migration and mutation rates.

In the studies of radionuclides in mosquitofish discussed above it was found that diversity increased in contaminated populations. This phenomenon may not be restricted to genotoxic stress, because there have been several papers which have suggested that environmental stress may lead to an increase of de novo variation through genetic recombination or mutations (Parsons 1987a,b, Cullis 1987). It is currently not known if increased genomic rearrangement is harmful, but it has the potential to harm by disrupting major adaptive gene complexes. In addition, in some cases transposistional rearrangements are maladaptive (eg. as in the case of oncogenes; Knudson 1986).

However, other studies have found that exposure to contaminants decreases diversity (see chapter 4 for a review). These discrepancies may be attributable to environmental or molecular origins. In environmental terms, these differences could be related to different types of contaminants (eg. genotoxic vs. non-genotoxic). In molecular terms, the type

of genetic marker may play a role. In most studies revealing decreased diversity, allozyme analysis was used. The genes examined in such studies are products of a small number of functional genes. In contrast, RAPD analysis may examine a larger segment of the genome, including non-coding regions, where rearrangement may be more likely. Discrepancies between the present studies and past studies of the effects of contamination on allozyme genotype frequencies may be due to allozyme variability: levels of heterozygosity in the present study are at least an order of magnitude lower than those reported in other studies. More studies need to be done using multiple genetic markers to reveal possible causes for these discrepancies.

In any case, decreased diversity may also have ecological ramifications. One measure of diversity is average heterozygosity, which can be measured at the individual (averaged between loci) or population level (averaged between individuals). For instance, a decrease in individual heterozygosity has been associated with decreased resistance to diseases (Ferguson and Drahushchak 1990, Wohlfarth 1985), growth rates (Wohlfarth 1985), and fertility (Leberg 1990). This would suggest that decreases in individual heterozygosity may affect population growth and recruitment. Another parameter of interest is population diversity. Effects of contaminants on variability are important because variability is associated with the adaptability of the population to

biotic (eg. disease or parasitism) or abiotic stressors. Furthermore, decreased heterozygosity may effect population performance, such as population growth rates (Leberg 1990)

Another reason why the effects of xenobiotics on genetic variation should also be of major concern is because these effects could be more refractory than individual-level effects. For example, if the source of pollution is removed from a population the metabolic and molecular processes of the organisms may return to normal within a few days or weeks, but genetic diversity may take many generations to return to a pre-contaminated state, if it ever does so at all. Specific genes or alleles may also be refractory after xenobiotic stressors are removed. For example, the genes for DDT resistance may persist in dipteran insect populations for years or decades after xenobiotic-induced selection is halted (Frölich and Würgler 1990).

Gene frequencies may be another useful population parameter for biological monitoring. It has been found that the frequencies of certain alleles can differ between contaminated and non-contaminated populations (Gillespie and Guttman 1988). For example, a change in gene frequencies downstream of an outfall compared to upstream may be indicative of pollutant-induced stress on the population. A related parameter is genetic distance (usually calculated from gene or allele frequencies). Suter (1990) has stated that a useful application of biomarkers in ecological risk assessment

is development of a model in which the level of effect is correlated to the level of contaminants in the environment. This may be applied to gene frequencies or genetic distance. For example, if the elevation of the frequency of a certain allele is indicative of contamination, then the frequency of this allele should decrease with decreasing concentrations of the contaminant - eg. for increasing distances downstream of an effluent. Also, if a site is known to be heavily contaminated, perhaps the genetic distances between this site and other sites with unknown histories of pollution could be calculated in order to assess which of the unknown sites is the most contaminated. A further use of genetic distance in ecotoxicology is to examine migration patterns in contaminated habitats.

Monitoring immigration and emigration to and from contaminated habitats is important for a number of reasons. First, it has been suggested above that populations may be able to adapt to particular contaminated environments. However, individuals migrating from non-contaminated sites would not have these adaptations, and adaptations to contaminated sites may be maladaptive in clean sites (see above). Thus environmental contamination may influence patterns of recruitment or dispersal. Secondly, contaminated organisms are potential routes of transport of contamination from contaminated to non-contaminated sites. Thus being able

to predict migration rates from population genetic data may be useful in formulating risk assessments.

The discussions above have focused on DNA damage and population genetics separately, but there are avenues of research which could combine the two. For instance, the studies in chapters 3-5 are significant to the study of genetic toxicology because they examine the relationship between effects at the molecular and population genetic level. There are also other studies which contribute to the understanding of this relationship. For example, a polymorphism in the cytochrome $P_{150}$1A1 gene was found in cancer-prone populations of fish but not in a reference population (Wirgin et al. 1991). Another study found that restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA and at an oncogene locus differed between tumor-prone and reference populations (Wirgin et al. 1990). Findings such as these could be useful for monitoring genotoxicant-stressed populations. In related studies, McMahon et al. (1989) and Wirgin et al. (1990) found mutations in oncogenes in liver tumors of fish exposed to genotoxicants. Similar studies are needed to determine if increase mutation rate of populations exposed to genotoxins is a general phenomenon. If this information is used in population genetic models it could be useful in assessing or predicting the population-level effects of genotoxic contamination.

The discussion above has addressed a number of ways in which population genetics can benefit ecological biomonitoring. However, in terms of ecological risk assessment, one must determine if changes in population genetic parameters are related to ecologically meaningful effects such as changes in population growth or productivity. Once such a relationship is established, perhaps monitoring changes in gene frequencies may be used as a surrogate for other types of ecological measures. For biomonitoring purposes, this has several advantages. For example, it has been stated that sampling population genotypes is more sensitive to levels of contamination and is fraught with less difficulty than direct census of populations (Benton and Guttman 1993). Furthermore, it has also been suggested that shifts in genotype may occur before changes in population densities or other ecological effects (Benton and Guttman 1993). Thus if prevention of ecological damage is one goal of environmental monitoring, then the population genetic monitoring may be more suitable for obtaining this goal than monitoring other types of population changes.

However, one caveat in monitoring population genetic structure is that just because the genetic structure differs between contaminated and reference populations, this does not necessarily mean that these changes are due to environmental contamination. In ecological risk assessment, assigning an ecological affect to a particular contaminant is facilitated

by the expression of biomarkers which are specific for that type of contamination (Suter 1990). The same can be true for population genetics: any change in population genetic parameters should be associated with a change in a particular biomarker or biomarkers to provide evidence for a cause and effect relationship. Further evidence would be a genotype-biomarker relationship. In other worlds, the magnitude of biomarker expression for individuals with the most common genotypes in contaminated environments should be greater (if the biomarker is indicative of detoxification or repair) or less (if the biomarker is indicative of a deleterious effect) than that of individuals with the less common genotypes. In order to provide convincing evidence that this sort of relationship is consistent and predictable, results should be obtained for both laboratory and field studies. Long-term laboratory or micro/mesocosm experiments examining changes in genotype upon xenobiotic exposure would provide additional evidence for this hypothesis.

A final consideration that merits attention is the relationship between single-species population effects and higher order effects dealing with multiple species. Because it is impossible to monitor every species and every interrelationship in the ecosystem, only a subset may be examined. For ecotoxicology investigations, comparative studies examining multiple species and interactions could be undertaken to establish a wide data base. For routine

monitoring purposes, economic and logistic factors constrain the number of species that can be examined. In this case, methods need to be developed which can use ecotoxicological information to accurately and rapidly assess ecological impacts using fewer numbers of species. In order to do this, one must focus on commonalities upon which generalizations can be made across species. Because DNA is one of the major commonalities of all living things, genetic ecotoxicology can provide valuable input toward this purpose. In fact, many of the major principles underlying molecular or population genetic processes are conserved across all five kingdoms of living organisms. This may facilitate extrapolation of ecological effects from models using one or a few species.

In conclusion, genetic ecotoxicology can be an important component in environmental biomonitoring programs. In order to be relevant to ecological risk assessment, such things as DNA damage or changes in population genetic structure must be related to higher-order effects. Finally, although reductionism of ecological processes into molecular components may not be practical or necessary in all cases, a thorough understanding of the relationship between genotype and environment can be a definite benefit to risk assessors and ecotoxicologists.

## CONCLUDING REMARKS

The results of this study support the primary focus of this dissertation: interpretation of toxicological data in an ecological setting. This was accomplished by examination of the relationship between laboratory and field studies and between individual and population-level responses. The results presented in this dissertation are important for three reasons: 1) They can provide ecological relevance to laboratory studies by facilitating extrapolation of laboratory results - which typically involve acute exposures and - to field situations - which involve chronic (usually life-long) exposures, 2) they provide verification and validation of field observations through the use of controlled laboratory experiments, and 3) they can provide a possible link between molecular and ecological responses to environmental contamination. Apart from providing valuable insights, these studies have also provided ample opportunity for future research into the mechanisms and possible ecological consequences of genotoxic contamination. Such studies in the future will no doubt be instrumental in evaluation of toxicological data in an ecological setting.

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