CHAPTER 41

Genetic Effects of Contaminant Exposure and Potential Impacts on Animal Populations

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“... it is important to take genetics into account in understanding if and how chemical contaminants impact populations.”

Peter Calow

41.1 INTRODUCTION

“Understanding changes to the genetic apparatus of an organism exposed to contaminants in the environment is essential to demonstrating an impact on parameters of ecological significance such as population effects. That field of environmental science that attempts to (a) identify changes in the genetic material of natural biota that may be induced by exposure to genotoxicants in their environment and (b) the consequences at various levels of biological organization (molecular, cellular, individual, population, etc.) that may result from this exposure is termed genetic ecotoxicology. Within genetic ecotoxicology, it is critical to realize that there are two possible classes of effects. First, there are effects that occur in the somatic or reproductive tissues of an organism. These effects are the result of direct exposure to a genotoxicant and have the potential to lead to somatic or heritable (genotoxicological) disease states. Another class of effects results indirectly from contaminant stress on a population and leads to alterations in the genetic makeup of populations, a process termed evolutionary toxicology. These latter types of effects alter the inclusive fitness of populations, such as by the reduction of genetic variability, and can potentially have profound impact on biomarker studies. For example, populations inhabiting contaminated and reference sites might be adapted to different environmental conditions and thus respond differently than expected in such studies.

With respect to genotoxicology (“a” above), it is now possible to identify molecular targets of genotoxicants with extreme sensitivity and to determine how chemical modifications of these targets affect function at a precise molecular level. For several reasons, approaches and studies related to “b” above are not as far advanced. First, a major challenge has been to develop assays with the sensitivity to demonstrate those subtle changes in the genetic material of organisms exposed to genotoxicants that may be genetic markers of population effects. However, recent advances in the discipline of molecular biology may provide the experimental tools with which to investigate those key biological mechanisms at the genetic level that regulate and limit responses of ecological relevance. Second, most studies performed in situ are often burdened by complicating environmental factors. Individual genetic variability within a population, population size, and exposure to complex mixtures are just a few of the many problems that must be addressed in order to interpret data generated by the sophisticated methodologies currently in use.

This chapter is divided into three main sections. The importance of understanding contaminant-induced DNA damage and related effects in relation to population-level studies is covered in Section 41.2, Genetic Effects. Section 41.3, Environmental Population Genetics, focuses mainly on new methodologies applicable to population genetic studies. Finally, Section 41.4, Case Histories, details several different investigations that provide insight on how chemical contaminants may impact populations.

41.2 GENETIC EFFECTS

41.2.1 Introduction

Within a cell, the structural integrity of the DNA molecule is in a constant state of flux between a functionally stable double-stranded entity without discontinuity and some intermediate, unstable state. This latter state is a transient phenomenon triggered by normal cellular processes. However,
these processes can be disrupted when exposure to a genotoxicant occurs, often with the concomitant loss of structural integrity of the DNA molecule. Some of the cellular responses that may be expressed after exposure to genotoxicants are given in Table 41.1. The organism’s inability (whether transient or permanent) to cope with loss of structural integrity provides the investigator the opportunity to detect environmental exposure to a genotoxicant. In addition, the occurrence of DNA damage provides a means to investigate the qualitative and quantitative relationships between the formation of DNA damage, subsequent DNA processing, appearance of deleterious lesions, and irreversible effects on reproduction and fitness. The reader is referred to the scientific literature for current reviews on this topic, in particular those of Shugart,\textsuperscript{10-12} Shugart et al.,\textsuperscript{13} Dixon and Wilson,\textsuperscript{14} and Wirigin and Theodorakis.\textsuperscript{15}

### 41.2.2 Types of DNA Modifications

A summary of some of the more common DNA structural modifications that occur when a genotoxicant becomes bioavailable and interacts with cellular DNA is recorded in Table 41.2. Two general classes of structural modifications can be inferred from the information contained therein. First, there are those modifications that identify the specific genotoxicant responsible for the structural modification. For example, ultraviolet light in the 290–300 nm range (UV-B) causes specific dimerization of pyrimidine bases within the DNA. Also, many chemicals, such as the polycyclic aromatic hydrocarbon (PAHs) and benzo[a]pyrene (BaP), can form an adduct with the

### Table 41.1 Cellular Responses after Exposure to Genotoxicants

<table>
<thead>
<tr>
<th>Biological Response</th>
<th>Expression in Cell</th>
<th>Temporal Occurrence$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detoxication</td>
<td>Protein induction: P450 enzyme system and metallothionine</td>
<td>Early</td>
</tr>
<tr>
<td>DNA Structural Modification</td>
<td>Covalent attachment of genotoxicant to DNA</td>
<td>Early</td>
</tr>
<tr>
<td>Adduct</td>
<td>Breakage of DNA phosphodiester linkages</td>
<td>Early</td>
</tr>
<tr>
<td>Strand Breaks</td>
<td>Hypomethylation and chemical modification of bases</td>
<td>Early/Middle</td>
</tr>
<tr>
<td>Base Modification</td>
<td>Induction of DNA repair enzymes</td>
<td>Early</td>
</tr>
<tr>
<td>Repair</td>
<td>Apoptosis</td>
<td>Early/Middle</td>
</tr>
<tr>
<td>Abnormal DNA</td>
<td>Chromosomal aberrations, micronuclei, aneuploidy, mutations</td>
<td>Middle/Late</td>
</tr>
<tr>
<td>Pathological Conditions</td>
<td>Neoplasia, tumors, and protein dysfunction</td>
<td>Late</td>
</tr>
</tbody>
</table>

$^a$ Temporal occurrence subsequent to exposure will depend on species of and type of genotoxicant. Early: hours to days; Middle: days to weeks/months; Late: weeks/months to years. (Source: From Shugart, L.R., Ecotoxicology, 9, 329, 2000. With permission.)

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### Table 41.2 DNA Structural Modifications Caused by Genotoxicants

<table>
<thead>
<tr>
<th>Genotoxicant</th>
<th>Type of Modification</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td>Thymine-Thymine Dimer</td>
<td>Dimerization of pyrimidine bases by UV-B light</td>
</tr>
<tr>
<td></td>
<td>Strand Breakage</td>
<td>Breakage of phosphodiester linkages due to formation of free radicals by ionizing radiation</td>
</tr>
<tr>
<td>Chemical</td>
<td>Adduct</td>
<td>Covalent attachment of genotoxicant to DNA molecule</td>
</tr>
<tr>
<td></td>
<td>Altered Bases</td>
<td>Chemical modification of existing bases</td>
</tr>
<tr>
<td></td>
<td>Abasic Sites</td>
<td>Loss of chemically unstable adduct or damaged base</td>
</tr>
<tr>
<td></td>
<td>Strand Breaks</td>
<td>Breakage of phosphodiester linkages due to formation of free radicals and abasic sites</td>
</tr>
<tr>
<td></td>
<td>Hypomethylated DNA</td>
<td>Improper postreplication</td>
</tr>
<tr>
<td></td>
<td>Mutation</td>
<td>Improper DNA repair</td>
</tr>
</tbody>
</table>

DNA. After metabolic activation, the BaP becomes covalently attached to the DNA. In both examples, the structural modification represents a specific fingerprint of the responsible genotoxicant. Second, there are those structural modifications that, although not specific to a particular genotoxicant, nevertheless suggest that exposure has occurred (e.g., breakage of the phosphodiester backbone of the DNA molecule). Strand breakage of the DNA can result when a genotoxicant produces free radical or forms an abasic site or sites. Also, many genotoxicants are known to interfere with normal DNA processing activities such as replication, methylation, and repair, which in turn may result in mutations (e.g., base addition/deletion). The detection of nonspecific structural modification (e.g., strand breaks, abasic sites, hypomethylation and mutations) may imply genotoxicant exposure, especially if the level or degree of these types of modification to the DNA molecule is not what might be anticipated (e.g., when compared to controls).

41.2.3 Detection of DNA Modifications

41.2.3.1 DNA Adducts

Detection of structural damage to DNA such as adducts is not an easy task, for several reasons. First, environmental genotoxicants are usually present at low concentrations and once they become bioavailable are readily detoxified. Therefore, the potential for in situ DNA damage is not high, and the amount that is found is often on the order of one adduct per 10^7 nucleotides or less. Second, until recently, the analytical technologies with the required selectivity and sensitivity to detect extremely low levels of DNA damage were not readily available. However, the application of modern techniques from the scientific disciplines of biochemistry and molecular biology has begun to alleviate this problem. In this regard, the possible application of DNA fingerprinting using PCR methodologies for the detection of structural DNA damaged, including adducts, caused by exposure to genotoxic environmental agents has been addressed.

The adverse health effects of most environmental chemicals are the result of their covalent binding to physiologically important receptor molecules. Identification of the interactive products with DNA, especially adducts, can represent the most direct and biologically relevant indicator of exposure to a genotoxicant. Numerous analytical methods to detect and quantify DNA adducts are available, with the 32P-postlabeling technique being the most used. The methodology is described in detail elsewhere. Because the salient features of this technique are sensitivity and selectivity, it is finding increased application in environmental monitoring studies where genotoxic contaminants exist. Lists of recent investigations that used the 32P-postlabeling technique to screen for DNA adducts in organisms taken from contaminated environments are available. It should be noted, however, that this technique is subject to problems that may interfere with the interpretation of the data generated. Several laboratories from Europe and North America are currently participating in a project to determine the extent of variability with this technique.

41.2.3.2 DNA Strand Breaks

Because both physical and chemical genotoxicants have the potential to cause DNA strand breaks, recent environmental studies have included this structural modification as an indicator of genotoxicant exposure. Several of the popular strand-break assays are based on the observation that under in vitro denaturation conditions of high pH, the rate of conversion of double-stranded DNA to the single-stranded moiety is proportional to the number of strand breaks in the DNA molecule. Among these are the alkaline elution assay, the alkaline unwinding assay, the gel electrophoresis method, and the comet assay. A list of investigations where these techniques have been applied are found in Shugart.
41.2.4 Cytogenetic Effects

DNA damage that is not corrected or is improperly processed may potentiate irreversible cellular
events\(^{4,15}\) that result in the appearance after cell division of abnormally processed DNA (e.g.,
chromosomal aberrations, micronuclei, somatic mutations etc., Table 41.1.).

Such cytogenetic effects result in alteration of the chromosome structure or chromosome
number. The traditional approach microscopically analyzes condensed chromosomes in metaphase
cells to determine the karyotype (i.e., number and appearance of chromosomes). Less laborious
and time consuming methods than karyological examination include micronucleus analysis and
detection of variation of DNA content among cells by flow cytometry. Micronuclei result from
acentric fragments of whole chromosomes that lag at anaphase and subsequently do not become
incorporated into either daughter nuclei after cell division but form their own small nucleus.\(^{28}\)
Flow cytometry is used to measure the differences in total DNA content among cells that result from the
unequal assortment of fragmented or rearranged chromosomal material after cell division.\(^{29}\) Obvi­
ously, cytogenetic, micronucleus, and flow cytometric analyses are measuring related phenomena.

41.2.5 Mutations

In addition to cytogenetic effects, faulty repair of genotoxic-induced DNA damage can result
in the occurrence of mutations in the DNA molecule (i.e., point mutations, additions/deletions,
translocations, etc.). In somatic tissue, mutations in oncogenes and tumor suppressor genes have
been associated with the initiation of chemical carcinogenesis. Because these genes are involved
in the regulation of cell growth, differentiation, and DNA repair, mutational events in these genes
can be correlated with aberrant cellular function, which can then be related to individual- and, it
is hoped, population-level effects.\(^{2,9}\) Virgin and Theodorakis\(^{15}\) discuss recent application of this
approach in relation to somatic and heritable effects of environmental contaminants on fish.

41.2.6 Protein Induction

The genetic apparatus of an organism can interact with a genotoxicant in a variety of ways that
may not result in structural modification to its DNA (Table 41.1.). The most common response is
that which results in the induction of a protein, or sets of proteins, involved with cellular detoxication
processes. For example, the organism may perceive\(^{7}\) the genotoxicant and modify its physiology,
as is found with the induction of the P4501A1 detoxication system.\(^{15,16,30}\) The induction of the
P4501A1 system can be detected by an increase in enzyme activity or enzyme protein, and the
magnitude of induction provides a measure of the degree of interaction of the inducing agent with
the aryl hydrocarbon hydroxylase receptor (Ah-receptor) in the cytoplasm of the exposed cell.

Metallothionein is a constitutive protein associated with the maintenance of homeostasis of the
trace metals zinc and copper. It is known to play a role in the detoxication of the genotoxic metals
cadmium and mercury, and upregulation of the metallothionein gene can serve as an early warning
signal of metal-induced toxicity.\(^{15,16}\)

A wide range of genotoxic agents can act as inducers (see discussion below).

41.2.7 Genotoxic Agents

A variety of contaminants can induce genotoxic responses. Some chemicals, including PAHs
and their nitrogenated or chlorinated derivatives, mycotoxins such as aflatoxins and related com­
ounds, and vinyl chloride typically exert their genotoxicity via formation of bulky adducts.\(^{18,31–35}\)
However, induction of oxidative damage may be a secondary mechanism of genotoxicity.\(^{36}\) A second
class is comprised of those genotoxic chemicals that cause derivatization of nucleotide bases via
transfer of methyl or ethyl moieties and includes the potent carcinogens diethylnitrosamine and
methylnitrosurea. Another class of genotoxic agents includes metals such as arsenic, cadmium, chromium, mercury, nickel, and lead. There are three possible mechanisms whereby metals may induce genotoxicity. First, some metals, chromium in particular, may adduct nucleotide bases. Second, there is growing evidence that metals may inhibit repair of DNA damage induced by chemicals or endogenous metabolism. Third, metals may increase levels of oxidative stress via redox cycling and Fenton reactions. There are also many organic chemicals that can potentiate genotoxicity via oxidative stress induction, and these include cyclic or aliphatic chlorinated hydrocarbons and several classes of pesticides.

Besides chemical agents, there are physical agents that can also lead to DNA damage, most notably several types of radiation. For example, ionizing radiation in the form of high-energy photons (γ- and x-rays), electrons (β-rays), or helium nuclei (α-rays) may be genotoxic. One mechanism by which ionizing radiation can induce DNA damage is via direct interaction of the radioactive particles with the DNA molecule. This can result in base alterations or breaks in the sugar phosphate backbone. Alternatively, the radioactive particles may interact with water or oxygen molecules, producing oxyradicals. These radicals may also produce base alterations or DNA strand breaks. Another type of physical genotoxicant is ultraviolet radiation, specifically UV-B. Irradiation of DNA with UV-B may result in covalent attachment of adjacent pyrimidine bases, resulting in so-called cyclobutane dimers. A secondary genotoxic effect of UV radiation is the production of oxyradicals. There have been suggestions that other types of electromagnetic radiation (e.g., radio and microwaves) and magnetic fields may also produce genotoxic effects, but this research is equivocal.

41.3 ENVIRONMENTAL POPULATION GENETICS

41.3.1 Introduction

Chemical contamination can cause population reduction by the effects of somatic and heritable mutations as well as nongenetic modes of toxicity. Although the original damage caused by chemical contaminants may be at the molecular level, there are emergent effects at the level of populations, such as the loss of genetic diversity, that are not predictable based solely on knowledge of the mechanism of toxicity of the chemical contaminants. In this regard, population genetic diversity has been proposed as a bioindicator of a population’s vulnerability to natural and anthropogenic stressors, as a record of genotypic variation in the population history and the effects of genotypic changes on the spatial distribution and abundance of populations in a geographic region.

Even though there is an extensive scientific literature in regards to classical Mendelian genetics, protein polymorphism, and DNA-marker studies in the field of population genetics, it is only recently that studies of the effects of pollution on population genetics have come to the forefront.

41.3.2 Genetic Markers

The oldest and most classical genetic marker is the phenotype, the visible traits or characters of individuals within a biological species. Phenotypic traits, such as mortality, developmental abnormalities, DNA strand breakage, physiology, and metabolism, stand as valid characters for population studies.

Another approach to population genetic analysis is to examine protein polymorphisms. An electrophoretic methodology, known as allozyme analysis, detects charge characteristics of enzymatic proteins produced by amino acid substitution. Allozyme analysis has been used in the past 20 years to assess the relationship between allozyme genotype and exposure to chemical compounds. The importance of the methodology to population genetic studies has been reviewed.
Recently, the application of DNA sequencing and the polymerase chain reaction (PCR)-based technologies has revolutionized the science of generating high-throughput genetic markers. New genetic-marker systems generated by the PCR methodology with applications to environmental genetics include RFLPs (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSRs (simple sequence repeats such as mini- and microsatellites), and AFLP (amplified fragment length polymorphism). These PCR-derived methods provide the potential to encompass large genomic regions, both coding and noncoding. A limited comparison of the capabilities of the various types of genetic markers is given in Table 41.3.

Since the various methodologies discussed above target different segments of the genome, possess differing resolution (Table 41.3), and involve varied operating and developmental costs, there is no single optimal technique (Table 41.4). Instead, methodological selection for environmental population genetic studies is guided by the problem under investigation.

### 41.4 CASE HISTORIES

This section is not intended to review all relevant literature but rather to present several case histories to demonstrate and illustrate (a) the types of techniques and methodologies that were applied to a particular study (b) that exposure to environmental contamination is a possible or likely cause of the population impacts observed, and (c) the kinds of genetic alterations seen or suspected to have occurred.

#### 41.4.1 Allozymes

Differences in allozyme allele frequencies between contaminated and reference sites have been found to occur in many species, and this may suggest that there is a selective advantage to certain genotypes over others in contaminated populations. For example, Gillespie and Guttman...
reported that some allozyme alleles were present at a higher frequency in contaminated stoneroller (Campostoma anomalum) populations than in reference populations. Fish with these alleles also had longer survival times when exposed to copper in the laboratory. In vitro enzymatic assays indicated that the enzymatic activity of these particular alleles was less inhibited by copper than that of the alleles that were more prevalent in noncontaminated populations. This not only linked genotype frequencies with survival (a component of selection) but also demonstrated a biochemical basis for differential susceptibility. On the other hand, selection may not act directly on the allozyme loci themselves, but rather these loci may be closely linked to other genes (e.g., detoxification enzymes, etc.) that impart a selective advantage.

In another series of studies, Newman et al. found that survival time of eastern mosquitofish (Gambusia holbrooki) exposed to heavy metals was correlated with allozyme genotype, particularly glucose–phosphate isomerase (GPI) alleles. Mulvey et al. (1995) went on to find that reproductive performance (number of gravid females and developing embryos per female) in these fish exposed to mercury was dependent on GPI genotype. Such differences in reproductive performance were in accordance with differences in survival among GPI genotypes. However, there was no evidence that such differences in survival and reproduction were related to differential susceptibility among GPI genotypes to enzymatic inhibition by mercury, either in vitro or in vivo.

Correlations between laboratory exposures and natural populations are not necessarily straightforward. Diamond et al. found that survival time of metal-exposed G. holbrooki was dependent upon allozyme genotype, but this pattern was not consistent among different populations or for fish collected from different years. Indeed, Lee et al. argued that correlations among broods or other subunits of a structured population may influence observed differences between polluted and reference populations. Consequently, the effectiveness of demonstrating contaminant-induced selection using allozyme may depend on the life-history characteristics, behavior, or local population structure.

In order to address other variables besides contaminant selection, Newman and Jagoe simulated mercury-driven selection for G. holbrooki GPI genotypes. They used simple and complex models to quantify the relative effects of viability selection, random genetic drift and migration on the GPI-allele frequencies, and sexual and fecundity selection. A simple suggested viability selection was a greater determinant than mortality-driven genetic drift, sexual selection, or fecundity selection. They also found that gene flow could abolish the effects of mercury selection on genetic differentiation among populations. In general, their model simulations indicated that changes in allele frequencies may reflect population-level effects of pollution, provided that the system under study is properly understood.

41.4.2 Puget Sound, Washington

It has been known for a long time that exposure to genotoxic agents may lead to neoplastic and preneoplastic lesions, and such patterns have been found in natural populations exposed to high levels of genotoxic contaminants, primarily in fish. Tumor incidence in fish and other aquatic organisms associated with exposure to genotoxic contaminants at a variety of sites throughout the United States including Boston Harbor, the Hudson River, Elizabeth River, Virginia, the Black River in Ohio, and the Great Lakes. However, perhaps one of the best-studied systems is in Puget Sound, Washington, which includes Eagle Harbor, a site heavily contaminated with PAH-laden creosote.

This system has been found to be contaminated with high levels of PAHs, and PAH adducts were associated with hepatic carcinomas in populations of English sole Pleuronectes vetulus. In addition, levels of hepatic DNA adducts have corresponded with known sediment or tissue-contaminant concentrations. In laboratory experiments using sole exposed to sediment collected from Eagle Harbor, PAH adducts demonstrated a linear dose-response function for both PAH concentration and length of exposure. In native fish populations, these adducts were associated with not only neoplastic lesions but also degenerative and preneoplastic lesions, and such lesions...
have shown significant associations with other biomarkers of PAH exposure and effect such as elevated cytochrome P450 and biliary PAH metabolite levels.\textsuperscript{75,76} Additionally, Reichert et al.\textsuperscript{76} have used a molecular epizootiological approach to provide definitive evidence that exposure to PAHs was the etiological agent in development of neoplasms. They found that levels of hepatic DNA adducts were a significant risk factor in the development of neoplasia in feral sole populations.

Further studies employ sequencing of the K-ras oncogene in order to study mutational events associated with neoplastic and preneoplastic lesions in this species.\textsuperscript{78} Hepatic lesion frequencies as well levels of DNA adducts and other PAH-indicative biomarkers were lower in fish collected after the cessation of discharge than were historical data collected when contaminant-generating activities were ongoing. The site has been capped with uncontaminated sediment, and these biomarker and histological endpoints are being used to assess the efficacy of remediation activates of Eagle Harbor.\textsuperscript{79}

Besides PAH adducts, other measures of DNA damage have also been examined in English sole from Puget Sound. For example, Malins and Haimanot\textsuperscript{80} found that oxidative DNA damage was highest in livers from sole that were tumorous from contaminated sites, least in sole livers from references areas, and intermediate in tumor-free fish from contaminated sites. This method also exposed positive associations between levels of oxidized bases and severity of preneoplastic and nonneoplastic lesions in livers of fish from the same area.\textsuperscript{81}

### 41.4.3 Sunfish

In 1987, the measurement of DNA strand breaks (see Section 41.2, Genetic Effects) in sunfish was implemented as a biological monitoring technique for environmental genotoxicity.\textsuperscript{82,83} Sunfish were initially collected and analyzed over a period of several years (1987–1992) from a contaminated stream (primarily mercury) and reference stream\textsuperscript{84} as part of a Biological Monitoring and Abatement Program for the U.S. Department of Energy (USDOE) in Oak Ridge, Tennessee. Analyses for DNA strand breaks in sunfish inhabiting these same streams were performed again in 1994–1995 by Nadig et al.\textsuperscript{85} and finally in 1997 by Theodorakis et al.\textsuperscript{86} Data collected indicated that the DNA structural integrity of sunfish from the reference stream was good (few DNA strand breaks) and remained relatively constant over the entire 10-year sampling period. However, levels of DNA strand breaks of sunfish from the contaminated stream fluctuated and varied with time of sampling. DNA damage was high in 1987 but started to decline in 1988. By 1992, the levels of DNA strand breaks were comparable to that found in the sunfish from the reference stream. The data from the 1994–1995 sampling period\textsuperscript{85} indicated a return to the high level of DNA strand breaks observed in 1987. However, by the 1997 sampling period,\textsuperscript{86} no significant DNA strand breakage was noted.

These data, in conjunction with other indicators of stress and toxicity,\textsuperscript{83} suggested that sunfish in the contaminated stream were being exposed to genotoxicants in a recurring manner. An improving aquatic environment, due to the effects of remedial actions implemented by the USDOE during the early years of sampling, was thought to be responsible for the diminution in DNA strand breakage that returned to normal levels observed in 1992. Subsequent release of contaminants into the stream after 1992 resulted in a return to the high levels of DNA strand breaks, which was documented in the 1994–1995 sampling period.\textsuperscript{85} Correction of the problem saw a return to the low levels of DNA strand breaks in the sunfish during the 1997 sampling period.\textsuperscript{86}

Theodorakis et al.\textsuperscript{86} extended the investigation of genetic effects in the sunfish to include both DNA strand breaks and chromosomal damage (measured by flow cytometry). In general, chromosomal damage in sunfish appeared to be correlated with mutagenicity of the sediment in the stream and was related to community-level responses (e.g., community diversity and percent pollution-tolerant species). Because responses at several levels of biological organization showed similar patterns of downstream effects, the authors suggested a causal relationship between contamination and observable biological effects.

The studies just described focused mainly on individual-level genetic effect (DNA strand breaks and chromosomal damage) from exposure of sunfish to contamination in their environment.
However, the studies of Nadig et al. extended this investigation beyond genetic effects at the individual level and examined potential alteration of population genetics. Using DNA markers produced by the RAPD technique (see Section 41.3.2, Genetic Markers), specific and unique genotypes were identified. Two measures of genetic diversity — the band-sharing index and the nucleon diversity index — showed that the sunfish from the contaminated and reference sites were different. Difference in genetic distance between populations was attributed to selection pressure of contaminants. This conclusion was supported by the finding that frequencies of certain unique genotypes in sunfish from the contaminated site correlated with a downstream gradient of mercury.

Taken together, these several studies show that sunfish were experiencing genotoxic stress as a result of exposure to contaminants in their environment. Analysis of DNA structural integrity reflected the level of insult from exposure at the time of sampling, while chromosomal damage data revealed the occurrence of irreversible cellular events as a result of this exposure. The observation that genetic diversity was altered in sunfish populations from contaminated sites compared with those from reference sites suggests that genetic selection occurred in the resident population and was probably due to contaminant effects.

The USDOE Biological Monitoring and Abatement Program in Oak Ridge has collected and archived a wealth of scientific information over the years on such topics as contaminant effects on biological species, waste management, and risk assessment. This program has, by design, the potential to advance our knowledge in the science of environmental population genetics, but to date it has been noticeably underutilized in this respect.

### 41.4.4 Mosquitofish

Beginning in 1992, a series of studies was initiated to determine the effects of ionizing radiation on DNA integrity and population genetics of western mosquitofish (Gambusia affinis) living in radionuclide-contaminated ponds on the Oak Ridge National Laboratory in Oak Ridge. In the first phase of these studies, DNA strand breakage was measured in mosquitofish exposed to ionizing radiation in situ. This was done by examination of four populations of mosquitofish, two from sites contaminated with radionuclides (Pond 3513 and White Oak Lake) and two from clean sites (Crystal Springs and Wolf Creek). The results of this study demonstrated that the double-stranded MML (median molecular length of DNA fragments detected by gel electrophoresis) of DNA of the fish from White Oak Lake and Pond 3513 was lower than from either of the two reference sites, indicating a higher degree of DNA strand breakage. Also, the single-stranded MML in the DNA of fish from Pond 3513 was lower than in any other population. It was also found that there was a direct correlation of DNA integrity (i.e., MML) with fecundity at least for single-stranded MML. There were no such relationships observed in the reference sites. These observations imply that resistance to DNA damage carries a fitness component, in that individuals that are better able to prevent or repair DNA damage are at a selective advantage in their environment. However, it could also be argued that this relationship is due to environmental factors. Therefore, the population genetics of these fish were examined to determine if this correlation had a genetic, rather than environmental, etiology.

In the next phase of these studies, the RAPD technique was employed in order to determine if the certain genotypes could impart a selective advantage in contaminated environments. A total of 142 RAPD bands were identified, and of these 16 were found to be present at a higher frequency in the contaminated sites relative to the reference sites ("contaminant-indicative bands"). The differences in frequency of the contaminant-indicative bands between contaminated and reference populations suggests that these bands may be genetic markers of loci that provide some sort of selective advantage in radionuclide-contaminated habitats. If this were true, it should be reflected in some component of fitness. To test this hypothesis, fecundity was examined in fish from each of the four populations with and without the contaminant-indicative bands. It was found that for seven of the contaminant-indicative bands in Pond 3513 and White Oak Lake, females that displayed...
these bands had a higher fecundity than those that did not. This was true for only one band in the Crystal Springs population. Another component of fitness is survival. Thus, if there is differential fitness between genotypes, then survival should be dependent on genotype for those fish exposed to radiation. To test this hypothesis, mosquitofish were collected from a noncontaminated pond and caged in another noncontaminated pond or in Pond 3513. It was found that for nine of the contaminant-indicative bands, the percent survival of fish with the band was greater than that for fish without the band.

These data imply that the contaminant-indicative bands may be genetic markers of loci that confer some sort of selective advantage in contaminated populations, in this case a higher degree of relative radioresistance. If the amount of DNA damage is a reflection of relative radioresistance, then the relative amount of DNA damage should be dependent on RAPD genotype. Therefore, the MMLs were compared for individuals with and without the contaminant-indicative bands. In order to do this, three separate experiments were performed. The first experiment used fish collected from the four populations described previously and used in determination of band frequencies. In the second experiment, 30 fish were collected from a noncontaminated pond and exposed to 20 Gy (approximately 12 min exposure time) of x-rays in the laboratory. The third experiment used the fish from the caging experiment described above. The results from these experiments indicated that for many of the contaminant-indicative bands, the fish that displayed the bands had higher DNA integrity than fish that did not display the bands.

If these bands are indeed genetic markers of loci that confer relative radioresistance, then this should also be reflected in other species exposed to radionuclides. To test this hypothesis, samples of a closely related species, *G. holbrooki*, were collected from two radionuclide-contaminated and two reference sites on the USDOE Savannah River Site (SRS). The population genetic structure of these mosquitofish was examined by the RAPD technique, using the same primers as were used in the Oak Ridge studies. It was revealed that the frequency of three RAPD markers (i.e., PCR-amplified DNA fragments) was greater in the DNA of fish from contaminated than the reference sites, and the frequency of two markers was greater in the reference than in the contaminated sites. These DNA fragments were the same size and amplified by the same PCR primers used in the ORNL study. Southern blot analysis, using labeled *G. affinis* RAPD bands as probes, revealed that the SRS *G. holbrooki* contaminant-indicative markers were homologous to the ORNL *G. affinis* contaminant-indicative markers.

If these RAPD fragments are genetic markers of selective advantage to fish in contaminated habitats, then it is possible that they are being amplified from a physiologically important locus. Thus, their DNA sequences may be conserved across taxa. To test this possibility, probes were made from 3 of the *G. affinis* RAPD primers described above. They were then hybridized to RAPD amplification products obtained from human, herring gull (*Larus argentatus*), and sea urchin (*Strongylocentrotus droebachiensis*) DNA, using the same RAPD primers as were used to produce the *G. affinis* RAPD bands described above. Southern blot analysis revealed that these markers were conserved in DNA sequence and molecular length in all species examined. The *G. affinis* bands were also cloned and sequenced, but the results of DNA sequencing efforts did not provide definitive evidence as to the identity of these loci. Although the identity of these bands is still unknown, the high degree of conservatism suggests that these loci might play an important role in molecular processes such as DNA repair, fitness, and survival.

These studies are significant for two reasons. First, genetic differences between populations may suggest selection for specific genotypes, but to validate this hypothesis, differential fitness and possible biochemical/molecular mechanisms for differential responses to toxicants must be shown. Second, integration of genotoxic or other molecular biomarkers (e.g., DNA strand breakage) into population genetic analyses could provide valuable insight as to the etiology and consequences of population genetic alterations. The concordance of all these results indicates that radiation exposure selects for certain genotypes, and the contaminant-indicative bands are markers of genes or other elements that confer a selective advantage in contaminated environments.
Due to ongoing restoration activities at the Oak Ridge National Laboratory, Pond 3513 is scheduled for remediation. To facilitate future scientific research initiatives with mosquitofish from this contaminated environment, samples were taken and are currently being maintained in laboratory aquaria at the Environmental Sciences division. Also, some carcasses have been archived and preserved in liquid nitrogen. Interested investigators should contact Dr. Mark Greeley.

41.4.5 Kangaroo Rats

The Nevada Test Site (NTS) is a nuclear weapons testing facility operated by the USDOE. Between 1951 and 1963, there were 105 aboveground tests of atomic weapons conducted at the NTS or its associated bombing range. In some sites, towers were located upon which bombs were placed for detonation. These ground-zero, or T, sites were used multiple times, and the surrounding areas received considerable radioactive contamination.

Theodorakis et al. conducted studies of the genotoxic effects of radiation from aboveground atomic bomb tests on Merriam’s kangaroo rat (Dipodomys merriami) at two of the T sites (T1 and T4). Initially, they used flow cytometry and the micronucleus assay to detect the somatic effects of radiation. These studies were inconclusive because, although cytogenetic analysis suggested genotoxic effects (means were higher in the contaminated sites than in the control sites), the differences were not statistically different. This is in spite of the fact that previous studies of heteromyid rodents exposed to chronic low-level radioactivity had revealed ecological effects.

Theodorakis et al. subsequently conducted molecular genetic analyses to better characterize the populations and search for population-level genetic effects. Two molecular genetic analyses — RAPDs and mtDNA control-region sequences — were employed in their study. Although the nuclear RAPDs did not reveal any differences among the four localities (two reference, R1, R2, and two ground-zero sites, T1 and T4), the maternally inherited mtDNA showed significant differences among populations. This was interpreted to mean that males disperse at a greater rate than females; thus, the nuclear markers reflect panmixia, but the maternal markers show population differentiation. This is consistent with behavioral studies on kangaroo rats in which males have been shown to disperse at a greater rate than females.

It was found that some mtDNA haplotypes were shared among sites (potential migrant haplotypes), and others were restricted to only a single site (potential resident haplotypes). Theodorakis et al. surmised that the unique haplotypes represented long-term residents and the shared haplotypes represented potential recent immigrants. To test this hypothesis, the flow-cytometry and micronucleus data were reanalyzed. It was found that when the animals with migrant haplotypes were excluded from the analysis, one of the contaminated sites had significantly increased DNA damage compared to one of the control sites. Furthermore, when animals from the contaminated sites were considered alone, individuals with resident haplotypes had significantly greater chromosome damage compared with animals with migrant haplotypes. This study shows that molecular genetic data can be used to better interpret biomarker data and that it is possible for genotoxic effects to be masked by high immigration from uncontaminated sites into contaminated sites. MtDNA is a potentially valuable genetic marker for differentiating among potential immigrants and residents.

The kangaroo rat data led Theodorakis et al. to hypothesize a specific demographic pattern of movement among populations. Reference area 2 (R2) proved to be significantly different in the biomarker analyses from contaminated T4; R1 and T1 were not different. They hypothesized that R1 was more likely to be exchanging migrants with the contaminated sites than was R2. This could be investigated by long-term field studies using mark-recapture techniques, but such data would take several years to obtain. To test this hypothesis using the genetics data, they conducted a phylogenetic analysis of the haplotypes and plotted the localities at which each haplotype occurred. Using the method of Slatkin and Madison, the hypothetical immigration events needed to explain the topology of the tree (which itself reflects the geneological history or genetic relatedness of the
haplotypes) was reconstructed. Using this analysis, Theodorakis et al.\textsuperscript{95} found that 27 migration events were needed to explain the tree, for 23 of which the direction of migration could be determined. Of these, 13 migration events involved movement of animals from the reference areas into the contaminated areas, and 6 involved migration from the contaminated areas into the reference areas. This is consistent with their conclusion that the shared haplotypes represented migrant individuals. The greatest number of migration events involved animals migrating from R1 $\rightarrow$ T4 ($n = 7$) and R1 $\rightarrow$ T1 ($n = 5$).

Therefore, this analysis supports the hypothesis that R1 serves as a significant source of migrant individuals for the contaminated areas. Furthermore, it changed the perception of the ecology of the ground-zero sites. The data are suggestive that the ground-zero sites are in fact sinks that are populated with a relatively high proportion of migrant individuals. For purposes of ecological risk assessment and ecotoxicological studies using biomarkers, the population genetic data in this case proved critical in obtaining a clear assessment of effects.

41.4.6 Bank Voiles

The meltdown at Chornobyl caused the worst nuclear power plant disaster, highly contaminating the area surrounding the reactor. Unfortunately, the impacts of this contamination upon wildlife remain largely undetermined. Two studies have been published, however, that shed light on the genetic effects of chronic exposure to radiation in natural populations near Chornobyl.

Matson et al.\textsuperscript{98} studied genetic effects of radiation on the bank vole, Clethrionomys glareolus, because it exhibits the highest internal levels of $^{134,137}$Cesium and $^{90}$Strontium among rodent species living in this area. Samples were collected over time from two contaminated sites, Glyboke Lake and the Red Forest (which has the highest levels of radiation of any area studied). Samples were also taken from one reference area, Oranoe, located outside the 30-km restriction zone. From these samples, a 291-base-pair region of the highly variable mtDNA control region, the D-loop, was sequenced and used to identify haplotypes. This study showed significantly higher genetic diversity in contaminated sites in comparison to the reference site.

Baker et al.\textsuperscript{99} continued the previous study, monitoring spatial and temporal dynamics of haplotype frequencies and genetic diversity. In addition to sampling the same reference and experimental sites used in Matson et al.,\textsuperscript{98} two additional reference sites were added, Chista and Nedanchichy (which has the lowest levels of radiation of any area studied). Sequential sampling of populations consistently showed significantly higher genetic diversity in experimental sites as compared to the reference sites. However, based on these data alone, the cause of increased variation in animals from contaminated sites was not determined. Two possible explanations to explain this observation were offered. First, increased genetic diversity could have resulted from mutations induced by exposure to radiation. Alternatively, it could be that the populations of C. glareolus were extirpated as a result of the meltdown of the reactor, causing an ecological sink. Consequently, multiple founder effects of animals emigrating from different areas resulted in an increase in genetic diversity in this area. To distinguish between these two hypotheses, monitoring studies are now being conducted at these sites. These studies include establishing pedigrees for resident bank voles using microsatellite analyses and monitoring changes in genetic diversity through time. Such data should reveal if new variants are evolving within the populations or are introduced by immigration.

41.5 SUMMARY

Laboratory studies have identified as genotoxic a large number of chemicals that are commonly found in contaminated environments. The adverse health effects of genotoxic chemicals on organisms often result from the consequence of direct DNA damage. While the majority of chemical-induced alterations to DNA are repaired, some are either not repaired or improperly repaired,
leading to mutations and changes in the genetic make-up of affected individuals. Genetic alterations in somatic tissue of an individual may not only have a number of immediate effects on the cells involved, but they may also provide an important clue as to the nature of the stress experienced by a population. Nevertheless, the most profound and long-lasting environmental effects occur at higher levels of biological organization.

When mutations occur in germ cells, they can potentially be passed to the offspring. Extrapolation of observations made at the somatic-cell level of biological organization to events occurring in germ cells in the same organism is difficult due to the inherent difference in sensitivity of these types of cells to genotoxics. Individuals carrying harmful mutations are often eliminated from the population due to a strong selection against less fit and less well-adapted individuals. However, the main concern for induced heritable mutations is that they will lower the reproductive output of an affected population since affected individuals have relatively low viability and fertility.

In addition, toxic chemicals, which do not interact directly with DNA, can also cause genetic effects on a population due to the selection or elimination of resistance or sensitive individuals in a population. Thus, adaptation can result in a narrowing of genetic diversity, which in turn is exasperated by associated ecological influences such as genetic drift, bottlenecks, and inbreeding, as well as the risk of producing the fixation of deleterious alleles.

Distinctive groups that differ genetically exist within natural wildlife populations. Variation in responses of organisms within these groups to toxic stress can be attributed in part to their genetic variations. In addition, contamination may influence the genetic composition of individuals within these populations and impose new or additional selection pressures. Stressed organisms are even more vulnerable to additional stressors, which may further jeopardize the survival of the population. Thus, the degree of genetic variation maintained by a population may be evidence of its capacity to survive future environmental alterations by tempering or modulating the stress-related effects of pollution. Genotypes that survive pollutant exposure may represent those individuals that are most tolerant to environmental stressors. For more detailed discussions on this topic, the reader should consult the scientific literature.

The work of Belfiore and Anderson on distinguishing between genetic alterations caused by natural processes and contaminants is especially relevant.

Genetic markers offer the most direct approach for measurement of genetic diversity. Two approaches pertaining to selection by anthropogenic stressors are found in the literature. The first is to identify genetic markers linked to either resistance or sensitivity to particular stressors or combination of stressors in select species, and the second is to employ a suite of genetic markers to examine population-level responses. Despite its limited resolution, allozyme analysis remains the simplest and most rapid technique for surveying genetic diversity in single-copy nuclear genes. The appeal of the PCR-based technologies is based on several factors including the simplicity of the procedure, the requirement for small amounts of DNA, and the potential to access many genetic loci. Employing genetic markers to assess genetic diversity of natural populations appears to be a promising and useful approach for determining the effects of environmental pollution on ecosystems.

Since the more significant ecological effects of contamination usually occur at the population or higher levels of biological organization, monitoring changes in population genetic structure will become a valuable component of ecological risk assessments. Research efforts in genetic ecotoxicology that deal with the ecological significance of exposure are rapidly expanding, as evidenced by the publication of a Special Issue on Environmental Population Genetics in the scientific journal Ecotoxicology. This special issue is a compilation of several current scientific research endeavors employing different approaches including classical allozyme analysis and genetic markers for studying the diversity (genetic variation) of population. These studies describe approaches and methodologies for the detection of stressor-induced effects on genetic diversity of populations, and several of them detail important case studies that demonstrate the usefulness of a particular approach to a given environmental problem.
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