MOLECULAR APPROACHES IN NATURAL RESOURCE CONSERVATION AND MANAGEMENT

Recent advances in molecular genetics and genomics have been embraced by many scientists in natural resource conservation. Today, several major conservation and management journals are using the "genetics" editors of this book to deal solely with the influx of manuscripts that employ molecular data. The editors have attempted to synthesize some of the major uses of molecular markers in natural resource management in a book targeted not only at scientists but also at individuals actively making conservation and management decisions. To that end, the text features contributors who are major figures in molecular ecology and evolution – many having published books of their own. The aim is to direct and distill the thoughts of these outstanding scientists by compiling compelling case histories in molecular ecology as they apply to natural resource management.

J. Andrew DeWoody is Professor of Genetics and University Faculty Scholar at Purdue University. He earned his MS in genetics at Texas A&M University and his PhD in zoology from Texas Tech University. His recent research in genetics, evolution, and ecology has been funded by organizations including the National Science Foundation, the U.S. Department of Agriculture (USDA) National Research Initiative, the Great Lakes Fishery Trust, and the National Geographic Society. His research is published in more than thirty-five journals, and he has served as Associate Editor for the *North American Journal of Fisheries Management*, the *Journal of Wildlife Management*, and *Genetica*.

John W. Bickham is Professor in the Department of Forestry and Natural Resources (FNR) and Director of the Center for the Environment at Purdue University. He received his MS in biology from the University of Dayton and his PhD in zoology from Texas Tech University. He was on the faculty of Texas A&M University's Department of Wildlife and Fisheries Sciences for thirty years. He has published more than two hundred articles in scientific journals in evolutionary genetics, including comparative cytogenetics, molecular systematics, molecular ecology, and ecotoxicology.

Charles H. Michler is the Fred M. van Eck Director of the Hardwood Tree Improvement and Regeneration Center at Purdue University and Site Director of the National Science Foundation Industry & University Cooperative Research Program's Center for Advanced Forest Systems. He earned his MS and PhD in horticulture, physiology, and biochemistry from The Ohio State University. He has published more than eighty-five scholarly works and has edited nine books and proceedings. He is Editor of *Plant Breeding Reviews* and Associate Editor of the *Journal of Forest Research*.

Krista M. Nichols is Assistant Professor, Departments of Biological Sciences and Forestry and Natural Resources at Purdue University. She received her MS in fisheries and wildlife from Michigan State University and her PhD in zoology from Washington State University. She was a National Research Council postdoctoral Fellow at the National Marine Fisheries Services, Northwest Fisheries Science Center. Dr. Nichols has published in the fields of ecotoxicology, genetics, and ecology.

Olin E. Rhodes, Jr., is Professor in the FNR and Director of the Interdisciplinary Center for Ecological Sustainability at Purdue University. He received his MS in wildlife biology from Clemson University and his PhD in wildlife ecology from Texas Tech University. He was named a Purdue University Faculty Scholar in 2006. He has published more than 135 scholarly works in ecology and genetics and has recently served as Associate Editor of the *Journal of Wildlife Management*.

Keith E. Woeste is a research molecular geneticist for the USDA Forest Service Northern Research Station Hardwood Tree Improvement and Regeneration Center and Adjunct Assistant Professor at Purdue University's FNR. He received an MDiv in theology from the Jesuit School of Theology at Berkeley, an MS in horticulture from the University of California–Davis, and his PhD in genetics from the University of California–Davis.

Molecular Approaches in Natural Resource Conservation and Management

Edited by

J. Andrew DeWoody
Purdue University

John W. Bickham
Purdue University

Charles H. Michler
Purdue University

Krista M. Nichols
Purdue University

Olin E. Rhodes, Jr.
Purdue University

Keith E. Woeste
Purdue University

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Contents

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Contributors

Gerard J. Allan
Department of Biological Sciences
Environmental Genetics and Genomics Facility
Northern Arizona University
Flagstaff, AZ

Giridhar N. R. Athrey
Department of Biology
University of Louisiana
Lafayette, LA

John C. Avise
Ecology and Evolutionary Biology
School of Biological Sciences
University of California
Irvine, CA

Joseph K. Bailey
Department of Biological Sciences and the Merriam-Powell Center for Environmental Research
Northern Arizona University
Flagstaff, AZ

Amy B. Baird
National Museum of Natural History – Naturalis
Leiden, The Netherlands

Randy K. Bangert
Biological Sciences
Idaho State University
Pocatello, ID

Robert C. Barbour
Cooperative Research Centre for Sustainable Production Forestry
School of Plant Science
University of Tasmania
Australia

Kelly R. Barr
Department of Biology
University of Louisiana
Lafayette, LA

Anbreen Bashir
Department of Biology
St. Louis University
St. Louis, MO

Dario Beraldi
Wild Evolution Group
Institute of Evolutionary Biology
School of Biological Sciences
University of Edinburgh
Edinburgh, UK

John W. Bickham
Department of Forestry and Natural Resources and Center for the Environment
Purdue University
West Lafayette, IN

Joseph D. Busch
Microbial Genetics and Genomics Center
Northern Arizona University
Flagstaff, AZ

Robert H. Devlin
Fisheries and Oceans Canada
West Vancouver, BC
Canada

J. Andrew DeWoody
Departments of Forestry and Natural Resources and Biological Sciences
Purdue University
West Lafayette, IN

Alexis Ducousso
UMR Biodiversité Gènes et Communautés
Institut National de la Recherche Agronomique
Cestas, France

Yousry A. El-Kassaby
Faculty of Forestry
University of British Columbia
Vancouver, BC
Canada

Julie R. Etterson
Department of Biology
University of Minnesota Duluth
Duluth, MN

Luke M. Evans
Department of Biological Sciences
Northern Arizona University
Flagstaff, AZ

Dylan G. Fischer
The Evergreen State College
Olympia, WA

Catherine A. Gehring
Department of Biological Sciences
Northern Arizona University
Flagstaff, AZ

Jake Gratten
Department of Animal and Plant Sciences
University of Sheffield
Sheffield, UK

Matthew C. Hale
Department of Biological Sciences
Purdue University
West Lafayette, IN

J. L. Hamrick
Department of Plant Biology
University of Georgia
Athens, GA

Richard G. Harrison
Department of Ecology and Evolutionary Biology
Cornell University
Ithaca, NY

David M. Hillis
Section of Integrative Biology
University of Texas
Austin, TX

Jeffrey D. Holland
Department of Entomology
Purdue University
West Lafayette, IN

Rebecca M. Holmstrom
Department of Biology
University of Minnesota Duluth
Duluth, MN

Rodney L. Honeycutt
Natural Science Division
Pepperdine University, Seaver College
Malibu, CA

Jamie A. Ivy
Department of Collections
San Diego Zoo
San Diego, CA

Sansanee Jamjod
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Antoine Kremer
UMR Biodiversité Gènes et Communautés
Institut National de la Recherche Agronomique
Cestas, France

Robert C. Lacy
Department of Conservation Science
Chicago Zoological Society
Brookfield, IL

Richard F. Lance
Environmental Lab
U.S. Army Engineer Research and Development Center
Vicksburg, MS

Emily K. Latch
Department of Biological Sciences
University of Wisconsin–Milwaukee
Milwaukee, WI

Peter W. Lawson
Conservation Biology Division
NOAA Fisheries
Northwest Fisheries Science Center
Newport, OR

Valérie Le Corre
UMR Biologie et Gestion des Adventices
Institut National de la Recherche Agronomique
Dijon, France

Paul L. Leberg
Department of Biology
University of Louisiana
Lafayette, LA

Carri J. LeRoy
The Evergreen State College
Olympia, WA

Wesley J. Leverich
Department of Biology
St. Louis University
St. Louis, MO

Denise L. Lindsay
Environmental Lab
U.S. Army Engineer Research and Development Center
Vicksburg, MS

Chanya Maneechote
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Marjorie Matocq
Department of Natural Resources and Environmental Science
University of Nevada
Reno, NV

Michelle M. McClure
Conservation Biology Division
NOAA Fisheries
Northwest Fisheries Science Center
Seattle, WA

Jennifer McCreight
Department of Forestry and Natural Resources
Purdue University
West Lafayette, IN

Paul McElhany
Conservation Biology Division
NOAA Fisheries
Northwest Fisheries Science Center
Seattle, WA

Allan F. McRae
Genetic Epidemiology Group
Queensland Institute of Medical Research
Herston, Australia

Charles H. Michler
Department of Forestry and Natural Resources and Hardwood Tree Improvement and Regeneration Center
Purdue University
West Lafayette, IN

David B. Neale
Department of Plant Sciences
University of California at Davis
Davis, CA

Krista M. Nichols
Departments of Forestry and Natural Resources and Biological Sciences
Purdue University
West Lafayette, IN

Sunisa Niruntrayakul
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Stephen R. Palumbi
Department of Biology
Hopkins Marine Station
Stanford University
Pacific Grove, CA

Josephine M. Pemberton
Wild Evolution Group
Institute of Evolutionary Biology
School of Biological Sciences
University of Edinburgh
Edinburgh, UK

Rémy J. Petit
UMR Biodiversité Gènes et Communautés
Institut National de la Recherche Agronomique
Cestas, France

Bradley M. Potts
Cooperative Research Centre for Sustainable Production Forestry
School of Plant Science
University of Tasmania
Australia

Amena Prommin
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Adirek Punyalue
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Tonapha Pusadee
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Benjavan Rerkasem
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Olin E. Rhodes, Jr.
Department of Forestry and Natural Resources
Purdue University
West Lafayette, IN

Kermit Ritland
Department of Forest Sciences
University of British Columbia
Vancouver, BC
Canada

Barbara Schaal
Department of Biology
Washington University
St. Louis, MO

Jennifer A. Schweitzer
Ecology and Evolutionary Biology
University of Tennessee
Knoxville, TN

Lee R. Shugart
LR Shugart and Associates, Inc.
Oak Ridge, TN

Theerasak Sintukhiew
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Jon Slate
Department of Animal and Plant Sciences
University of Sheffield
Sheffield, UK

Fredrik L. Sundström
Fisheries and Oceans Canada
West Vancouver, BC
Canada

Athitya Suta
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Chris W. Theodorakis
Biology Department
Southern Illinois University at Edwardsville
Edwardsville, IL

Peter M. Visscher
Queensland Institute of Medical Research
Royal Brisbane Hospital
Queensland, Australia

Thomas C. Wainwright
Fish Ecology Division
NOAA Fisheries
Northwest Fisheries Science Center
Newport, OR

Lisette Waits
Fish and Wildlife Resources
University of Idaho
Moscow, ID

Robin S. Waples
Conservation Biology Division
NOAA Fisheries
Northwest Fisheries Science Center
Seattle, WA

Peter M. Waser
Department of Biological Sciences
Purdue University
West Lafayette, IN

David J. Weston
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN

Thomas G. Whitham
Department of Biological Sciences and Merriam-Powell Center for Environmental Research
Northern Arizona University
Flagstaff, AZ

Alastair J. Wilson
Institute of Evolutionary Biology
School of Biological Sciences
University of Edinburgh
Edinburgh, UK

Keith E. Woeste
Department of Forestry and Natural Resources and Hardwood Tree Improvement and Regeneration Center
Purdue University
West Lafayette, IN

Anupong Wongtamee
Faculty of Agriculture
Chiang Mai University
Chiang Mai, Thailand

Lisa Worthen
Department of Forestry and Natural Resources
Purdue University
West Lafayette, IN

Stan D. Wullschleger
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN

Kelly R. Zamudio
Department of Ecology and Evolutionary Biology
Cornell University
Ithaca, NY

Preface

The world would be a wonderful place if our natural resources (e.g., forests, fish, and wildlife) needed no management and conservation was not a concern. In a world with a global human population approaching 7 billion and where most developed nations overconsume these resources, however, conservation is a concern and management is necessary for sustainable use. Historically, natural resource management strategies were determined by the collection and interpretation of basic field data. Today, as challenges to the sustainability and conservation of our natural resources arise, managers often need data that cannot be acquired using conventional methods. For example, a natural resource manager might want to know the number of successful breeders in a population or if genetic variation was being depleted because of a management practice. Traditional field craft alone cannot directly address such questions, but the answers can be determined with some precision if the field work is coupled with modern molecular genetic techniques.

Molecules can enlighten us about biological attributes that are virtually impossible to observe in the field (Avise 2004). Parentage analysis is one such arena in which genetic data can inform management practices (DeWoody 2005), but there are a host of others. For example, molecular data have revealed deep evolutionary splits in stocks at one time thought to be homogeneous. This finding has concomitant management implications (Hoffman et al. 2006). Similarly, molecules can enlighten us about biologies that are virtually impossible to observe in the field, such as pollen flow (Hamrick, this volume) or the physiology of migration (Nichols et al. 2008).

Recent advances in molecular genetics and genomics have been embraced by many scientists in natural resource conservation. Today, several major conservation and management journals (e.g., *Journal of Wildlife Management, North American Journal of Fisheries Management, Plant Breeding Reviews*) are now using "genetics" editors to deal solely with the influx of manuscripts that employ molecular data. We have attempted to synthesize some of the major uses of molecular markers in natural resource management in a book targeted not only at scientists but also at individuals actively making conservation and management decisions. To that end, we have identified contributors who are major figures in molecular ecology and evolution; many have published books of their own. Our aim has been to direct and distill the thoughts of these outstanding

scientists by compiling compelling case histories in molecular ecology as they apply to natural resource management.

Clearly, we hope this book will appeal to academics interested in conservation genetics, molecular ecology, and the quantitative genetics of wild organisms. We think this book could be used as an educational tool – as a text for graduate ecology/genetics courses but also, perhaps, in advanced undergraduate courses. Furthermore, we hope this book will be useful to audiences in natural resource management, education, and research by clarifying how genetic approaches can be used to answer resource-related questions.

ABOUT THE EDITORS

Our collective expertise spans from molecular population genetics in the wild to genomics and quantitative genetics of managed or cultured species. We all study the genetics of natural resources, however, and we find that similar issues arise in wildlife, forestry, and fisheries. For example, when the forest geneticists began asking how many sires contributed pollen to a nut-bearing hardwood tree, it turns out that fisheries geneticists had already studied this problem from the perspective of a male fish guarding a nest full of developing embryos, and they had created computer programs to estimate the number of parents contributing gametes to a nest (DeWoody et al. 2000). Another such intersection of research across disciplines lies in the study of genetic processes in small populations; the same conceptual and analytical approaches being used to elucidate the genetic consequences of wildlife reintroductions (Latch & Rhodes 2005) are employed to evaluate genetic diversity in hardwood tree species subjected to severe habitat fragmentation (Victory et al. 2006). Our desire to produce a book stems from our mutual interests in understanding how molecular genetics can be used to inform and improve natural resource management.

In addition to our research interests, we teach several courses that directly pertain to this book. These courses include *Conservation Genetics* (DeWoody), *Molecular Ecology and Evolution* (DeWoody), and *Evolutionary Quantitative Genetics* (Nichols). Furthermore, several of us (DeWoody, Michler, Rhodes) have served as "genetics" editors for conservation and management journals, including *Journal of Wildlife Management, North American Journal of Fisheries Management*, and *Plant Breeding Reviews*.

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Book contributors at an October 2008 meeting, held at the John S. Wright Forestry Center (Purdue University). Row 1: Krista Nichols, Kelly Zamudio, Charles Michler, Yousry El-Kassaby, Tom Whitham, Jamie Ivy, Emily Latch, Lisette Waits, and Marjorie Matocq. Row 2: Lee Shugart, Dave Neale, Dave Hillis, John Avise, Andrew DeWoody, Robin Waples, Rodney Honeycutt, Paul Leberg, and John Bickham. Row 3: Kermit Ritland, Antoine Kremer, Stan Wullschleger, Keith Woeste, Peter Waser, Jim Hamrick, Gene Rhodes, and John Patton. Photo credit: Caleb D. Phillips. *See Color Plate I*.

individual chapters and boxes, and we trust that this book has been enhanced by their efforts.

This volume was largely possible because of the financial and logistical support of the Department of Forestry and Natural Resources at Purdue University. In particular, the department sponsored an October 2008 meeting at Purdue where many of the book contributors congregated for three days of scientific discourse and fellowship before finalizing their respective chapters or boxes.

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13 Evolutionary toxicology

Lee R. Shugart, Chris W. Theodorakis, and John W. Bickham

It is clear that scientific studies that investigate the influence of environmental pollutants on evolutionary processes are applicable to all species. The discussion here, however, deals with organisms other than humans, and it recognizes, of course, that distinctions, at the molecular level, between toxic effects to humans and to other species often are not discretely distinguishable. The important distinction to be made here is that studies of the effects of environmental contaminants on human health are ultimately concerned with the health of individuals, whereas studies that deal with natural populations of wildlife or other organisms focus on the well-being of populations.

Evolutionary toxicology is the study of the effects of pollutants on the genetics of natural populations (Bickham & Smolen 1994; Bickham et al. 2000; Matson et al. 2006). The emergence of evolutionary toxicology as a new field of scientific investigation was noted in the early 1990s at the Napa Conference on Genetic and Molecular Ecotoxicology held at Yountville, California, in October 1993 (Bickham & Smolen 1994). The stated goal of this new endeavor was to identify environmental pollutants that influence evolutionary processes and to quantify the extent of their effects. The procedures and techniques of modern molecular biology to population genetics were invoked to accomplish this goal.

Evolution includes the change in inherited traits of a population from one generation to the next. Natural factors that drive genetic diversity are gene flow, mutation, natural selection, bottlenecks, and genetic drift (Belfiore & Anderson 2001; Staton et al. 2001). Mutations to genes are changes in the nucleotide sequence of an organism's genetic material that can produce new or altered traits in individuals. These changes, in turn, can produce disease, illness, or susceptibility in numerous ways. Gene flow as a result of interactions with immigrating individuals into a population can cause a variety of effects, including increased genetic diversity, homogenization of genetic patterns across populations, and even hybridization among species. Mutations may be spontaneous or induced by exposure to genotoxins (i.e., ultraviolet and high-energy radiation or certain chemicals). Both processes serve to increase genetic diversity through the introduction of new alleles into a population. Reduced genetic variability can be

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caused by bottlenecks, which are drastic reductions in a population's size due to natural or manmade events, combined with genetic drift, which produces the random elimination of alleles from a small population.

Chemical toxicants and radiation have the potential to affect genetic systems in two general ways. First, somatic effects result from the direct interaction between a mutagen and the DNA of an organism. These interactions can occur in any cell in the body, including somatic and reproductive tissues, and there is a wide range of consequences for the health and reproductive success of the organism. Environmental pollutants that directly change the integrity of an organism's DNA with subsequent adverse effects are designated genotoxicants. Moreover, genotoxicants can be either natural or man-made mutagens that an organism encounters in its environment. Conversely, environmental pollutants that do not directly interact with the DNA are nongenotoxicants.

Second, population genetic effects can be caused by genotoxicants or nongenotoxicants that have a sufficiently profound effect on survival and reproduction to create a measurable impact on genetic patterns. This latter class of environmental effects results from impaired reproductive success that leads to selection or other indirect changes in population genetic structure or diversity (Rose & Anderson 2005). These effects include the reduction of genetic diversity that results from population bottlenecks with subsequent genetic drift that typically eliminates rare or low-frequency alleles. Reduction in genetic diversity also results from selection at survivorship loci that allows certain individuals to live and successfully reproduce in the contaminated environment with the concomitant loss of the alleles that allow successful reproduction in pristine sites. An increase in genetic diversity results when pollutants induce genetic mutations that create new alleles found only at the contaminated sites. Finally, contaminated environments might also create ecological sinks that alter the patterns of gene-flow. These patterns represent emergent effects and are not necessarily predictable from a knowledge of the toxicity mechanisms of the contaminants. In other words, genotoxicants like polycyclic aromatic hydrocarbons (PAHs), ionizing radiation, and certain heavy metals can have population effects similar to those of nongenotoxicants like many organochlorines and other classes of compounds that act as endocrine disruptors. Notwithstanding the fact that the actions of these compounds at the molecular level are entirely different, their emergent population effects might be similar.

DISRUPTION OF GENE STRUCTURE BY ENVIRONMENTAL POLLUTANTS

Human knowledge about toxic effects of chemicals dates back to early history and the use of plant and animal extracts in hunting (Egyptian papyrus roll ~1,500 BC). A milestone in the study of adverse effects of chemicals on living organisms was the observation by Paracelsus in the sixteenth century that there is a link between the dose of a poison and its biological effect. The enormous industrial and economic development that occurred after World War II resulted in an unprecedented release of untreated anthropogenic chemicals into the environment. Nevertheless, at that time, the idea that chemicals present in the

environment might be dangerous to human health was essentially nonexistent. In the 1960s, however, our thinking that chemicals posed no adverse environmental risks began to change (Carson 1962). In the 1970s, toxicological investigations expanded to the study of pollutant effects on populations and entire ecosystems with the emergence of ecotoxicology as a prominent new scientific discipline (Newman 1998; Walker et al. 2006). Today, the detrimental health effects of many of the chemicals found in the environment are well documented, and the biological mechanisms responsible for their toxicity are under extensive investigation (Yu 2005).

Biomarkers

The term *biomarker* is currently defined as a biological response expressed at or below the organism level of biological organization and which can be related to "the exposure to" or "the effect of" chemicals in the exposed organisms (McCarthy & Shugart 1990; Peakall & Shugart 1993).

Because the health of the organism can be affected on exposure to a chemical, toxicological research often focused on those biological measures that might be indicative of toxicity (Depledge et al. 1993). The rationale that the identification and use of sufficiently sensitive biological responses of toxicity (biomarkers) may be useful tools of toxicity assessment hinged on an important paradigm of ecotoxicology: "organisms exposed to chemicals may demonstrate a state of toxicity which is related to the capacity of the organism to withstand the toxic stress." Toxicity is initiated upon exposure with the interaction of environmental chemicals and cellular biomolecules. The potential for the initial toxic effect to cascade through the organism starting at the biochemical level and progressing to the organism level will depend on the resiliency of the individual. Subsequently, deleterious effects may be observed at the population level of biological organization. Thus, biomarkers are viewed as measurable biological responses in an organism exposed to stressors present in its environment and, when appropriately applied, certain cause–effect relationships can be inferred. In some instances, they are predictors of the adverse consequences of that exposure.

Several key workshops, symposia, and conferences were held during the late 1980s and through the 1990s to identify and articulate many of the important concepts associated with biomarkers (Table 13–1). From a historical viewpoint, these various venues were instrumental in establishing the framework, as currently practiced by the scientific community, for the implementation of biomarkers to evaluate environmental toxicity.

Genetic ecotoxicology

Background

Genetic ecotoxicology is the study of the effects of substances or agents (e.g., chemicals and radiation) on the genetic material of natural populations, and subsequent related population- and community-level responses (Anderson et al. 1994). It is a complex discipline that integrates knowledge from diverse fields

Table 13–1. Important workshops, symposia, and conferences on the development, application, and evaluation of biological markers in environmental health

Venue	Topic	Reference
ACS Symposium 1990, Los Angeles, CA, Fall 1988	Biomarkers of environmental contamination	McCarthy & Shugart 1990
8th Pellston Workshop, Keystone, CO, July 23–28, 1989	Biomarkers: Biochemical, physiological, and histological markers of anthropogenic stress	Huggett et al. 1992
NATO Advanced Research Workshop, Netherlands Institute of Sea Research, Texel, The Netherlands, May 12–17, 1991	Biomarkers: Research and application in the assessment of environmental health	Peakall & Shugart 1993
International Workshop, Certosa di Pontignano, Siena, Italy, May 25–27, 1992	Nondestructive biomarkers in vertebrates	Fossi & Leonzio 1994
NATO Advanced Research Workshop, Luso, Portugal, June 1–5, 1992	Use of biomarkers in assessing health and environmental impacts of chemical pollutants	Travis 1993
National Institute of Environmental Health Sciences (NIEHS) Conference, Yountville, CA, October 12–15, 1993	Napa conference on genetic and molecular ecotoxicology	Anderson et al. 1994
NIEHS Study Group, Spring and Fall, 1994	Ecotoxicity and human health: A biological approach to environmental remediation	de Serres & Bloom 1995
North Atlantic Treaty Organization (NATO) Advanced Research Workshop, Cieszyn, Poland, September 21–25, 1997	Biomarkers: A pragmatic basis for remediation of severe pollution in Eastern Europe	Peakall et al. 1999

such as genetic toxicology, ecology, molecular biology, and population genetics (Forbes 1998; Newman and Jagoe 1996).

The basic scientific principles representative of genetic ecotoxicology emerged from observations made in early studies in radiobiology (Rose & Anderson 2005). In the 1950s, radiation exposure was shown to alter the development, growth, and reproduction of mammals, fishes, and invertebrates. Growth retardation, suppression of cell division, and modified cell differentiation were detected in radiation-exposed organisms. Radiation exposure was also linked to gonad sterility as well as reduced fecundity, hatching success, and fertilization success in both vertebrate and invertebrate species. By the 1960s, congenital and developmental abnormalities that occurred in animals exposed to radiation were shown to be associated with chromosome damage and mutations. Similar pathological responses were noted upon exposure to certain chemicals (genotoxicants).

As a subfield of ecotoxicology, genetic ecotoxicology can trace its origin to the search for biological responses (biomarkers) that were indicators of genotoxicity (McCarthy & Shugart 1990; Peakall & Shugart 1993). Scientists were acutely aware

that changes in certain biological responses (biomarkers) of organisms could be attributed to the toxic effects of environmental pollutants and exploited these observations in their effort to document environmental health issues (Stein et al. 1992). Specific genetic damage, which was shown to be initiated by the interaction of a genotoxicant with the DNA molecule, became a biomarker for studies in genetic ecotoxicology (Varanasi et al. 1981; Shugart et al. 1992; Shugart & Theodorakis 1998). Early application (e.g., Martineau et al. 1988) of DNA damage as a biomarker for exposure to environmental genotoxicants was greatly facilitated by the availability of sensitive and specific analytical methods that had been developed for human health studies (Rahn et al. 1982).

Significance of early studies with DNA adducts

DNA adduct is the term used to describe the moiety that results from the covalent bonding of a chemical to the DNA molecule. DNA adducts can occur in living organisms upon exposure to chemicals in their environment. The base guanine is by far the most prevalent target, although adducts have been reported for all bases. DNA adduct levels, measured at any point in time, reflect tissue-specific rates of adduct formation and removal, which depend on chemical activation, DNA repair, adduct instability, and tissue turnover. Adduct formation in humans appears to be indicative of molecular dosimetry and suggestive of increased human cancer risk. Nevertheless, this relationship has been defined for only a few carcinogens and remains a compelling challenge for future investigations. The chemical structure of most DNA adduct moieties identified in the early 1990s was characterized by chemically specific techniques using mass, fluorescence, and nuclear magnetic resonance spectrometry (Hemminki et al. 1994). Such information was invaluable in determining the stereo- and region-selectivity of enzymatic reactions involving chemical activation.

The early studies on DNA-adduct detection and identification were part of a broad investigation of chemical carcinogenesis in humans. As early as the late 1940s, it was known that certain chemicals, upon ingestion by the test animal, would bind to cellular macromolecules (Miller & Miller 1947). Two decades later, more detailed studies showed that PAHs could bind to mouse-skin DNA (Brookes & Lawley 1964; Boshman & Heidelberger 1967). Subsequent investigations in animals exposed to potential carcinogenic chemicals focused on two main research areas: 1) metabolism, and 2) reaction with cellular macromolecules. It was soon realized that these two areas were linked because these hydrocarbons undergo metabolic activation within cells to intermediates that react covalently with nucleic acids (Grover & Sims 1968; Brookes & Heidelberger 1969; Gelboin 1969). It should be noted that PAHs are ubiquitous in nature and, for experimental design, many can be obtained in pure form. Some individual PAHs (i.e., dibenz[a,h]anthracene and benzo[a]pyrene [BaP]) are known to cause cancer in experimental animals. For these reasons, and especially because the cellular mechanisms for DNA-adduct formation for all chemicals tested were shown to be similar, this class of chemicals, in particular BaP, became the "gold standard" for DNA adduct studies with respect to metabolic activation, covalent binding to DNA, and detection methodologies (Phillips 1990).

Investigations concerned with the metabolic activation of chemicals showed that the metabolism of chemicals is similar in most organisms and occurs via

a cellular detoxication/toxication system in steps: Phase I (biotransformation) and Phase II (conjugation) reactions. The Phase I system involves oxidation by various monooxygenase reactions including epoxidation, hydroxylation, and dealkylation and is catalyzed by the cytochrome P-450 monooxygenase- or mixed-function oxidase (MFO) enzyme system, which are iron-containing hemoproteins. The terminal component of this system, cytochrome P-450, exists in multiple forms. The resulting products of Phase I metabolism may be converted to dihydrodiols and/or conjugated (Phase II) with glutathione, glucuronic acid, or sulfate. An important feature of the MFO enzyme system is that the activities and concentrations of specific isoenzymes of cytochrome P-450 can be induced by exposure to xenobiotics. Paradoxically, during the course of metabolism, certain xenobiotics may form reactive electrophilic intermediates, which are at higher energy contents than the parent compounds and thus have the potential to interact covalently with nucleophilic sites in DNA, ribonucleic acid (RNA), and protein. The metabolism and disposition of PAHs in aquatic organisms (in particular, fish) were well understood in the 1980s (Stegeman 1981; Varanasi et al. 1981, 1989).

Sensitive and specific methods for DNA-adduct measurements were in place by the early 1990s (Phillips 1990). The most frequently used methods included immunoassays and immunohistochemistry (Poirier 1981; Kriek et al. 1984) using adduct-specific antisera, electrochemical detection (Yamamoto & Ames 1987), fluorescence (Rahn et al. 1982; Jeffrey 1991), mass spectrometry (Fedtke & Swenberg 1991), and ^{32}P-postlabeling (Randerath et al. 1981). These methodologies, which were developed primarily for human and experimental animal investigations, were applied to studies with environmental species (Varanasi et al. 1986; Shugart 1998).

Future studies in genetic ecotoxicology

The field of genetic ecotoxicology has expanded significantly in the past several years to include investigations that focus on toxic exposure and subsequent population-level effects with evolutionary implications. The integration of population genetics with genetic ecotoxicology can provide a useful approach for evaluating the long-term and high-order effects of environmental genotoxic pollutants (Würgler & Kramers 1992; Shugart et al. 2003). Biomarkers of genotoxicity are measures of individual responses to environmental pollutants that will improve assessment of organismal fitness (Bickham & Smolen 1994; Theodorakis & Shugart 1999; Theodorakis & Wirgin 2002; Matson et al. 2006; Theodorakis et al. 2006). Thus, to differentiate between evolutionary toxicology and genetic ecotoxicology, it should be noted that the former is essentially the field of science that describes how organisms adapt to polluted environments, whereas the latter describes the genotoxic effects of environmental pollutants from the molecular level to the population-genetic level (Leet and Richards in press).

Population-level consequences

Change in gene frequency is at the heart of adaptation, speciation, and evolution. Bickham and colleagues (2000) and Rose and Anderson (2005) provide

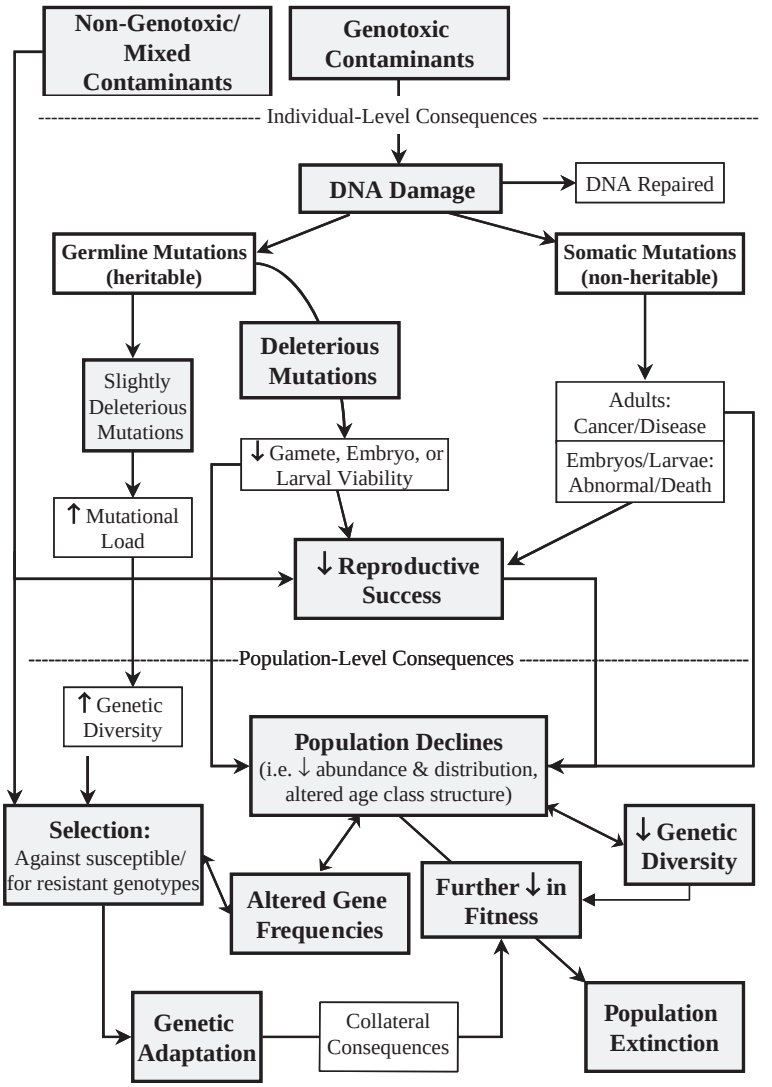


Figure 13–1: Exposure to environmental pollutants and individual- and population-level consequences. By permission: Rose WL, Anderson S I (2005) Genetic ecotoxicology. In: *Encyclopedia of Toxicology*, 2nd edn. (ed. Wexler P), pp. 126–132. Elsevier Ltd., Oxford, UK.

thorough discussions of the complex processes and pathways by which environmental pollutants can interfere with the genetic makeup of individuals and have consequences at the population level.

Figure 13–1 (taken from Rose & Anderson 2005) details a model that relates pollutant (usually a toxic chemical) exposure to population-level consequences. The model incorporates the potential for a pollutant to act as a genotoxicant or a nongenotoxicant.

Genotoxicants act by disrupting gene structure at the cellular and molecular levels either by directly causing DNA damage or via cellular mechanisms, including induction of oxidative stress, inhibition of DNA repair (which eventually can result in DNA damage), and chromosomal breaks or rearrangements. DNA

damage not repaired or repaired incorrectly can lead to adverse health effects, or stress, on the individual (Shugart 1996, 1998). These effects are depicted in Figure 13–1 as individual-level consequences to genotoxicant exposure and can be responsible for population-level consequences such as reproductive impairment or high mortality rates. Population-level consequences, in turn, can cause demographic effects that result in reduction of genetic variability in populations or the alteration of gene-flow patterns. Thus, although a genotoxicant initially exerts its effects at the molecular level, it has the potential to initiate a cascade of responses that may influence evolutionary processes, including adaptation and extinction.

Nongenotoxic pollutants are toxic substances that do not alter the genetic material of the exposed organism (Colborn 1994). They may impair reproductive success or lead to selection and indirect changes in population structure (Figure 13–1). A frequently cited example of a nongenotoxic pollutant is dichlorodiphenyltrichloroethane (DDT), a synthetic chemical used as an agricultural insecticide. This chemical and its breakdown products are toxic to embryos and disrupt calcium absorption, resulting in eggshell thinning in birds. Population reduction in several exposed species of birds was due to DDT exposure. Many environmental pollutants, including DDT, are classified as endocrine disruptors, which are exogenous substances that act like hormones in the endocrine system and disrupt the physiologic function of endogenous hormones. Endocrine disruptors are linked to several different adverse biological effects that can affect survival in animals (Colborn et al. 1993). One such way in which environmental pollutants, including endocrine disruptors, can affect survival is by altering gene expression. Epigenetics is the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms. Currently, the term is used to describe those processes that establish heritable states of gene expression without altering the DNA sequence of the organism. These processes result in gene expression that is stable between cell division and sometimes between generations. The latter epigenetic phenomenon is referred to as "transgenerational inheritance" (Anway et al. 2005).

Thus, both genotoxic and nongenotoxic pollutants have the capability of impacting population genetic processes by a variety of mechanisms and pathways. The complexities of molecular, cellular, physiological, and reproductive processes that are involved in genetic ecotoxicology belie the relative simplicity of higher-order effects expressed at the population-genetic or evolutionary level. Notwithstanding the fact that recent advances in ecotoxicology have included the discovery of broad new classes of toxic effects (i.e., endocrine disruptors) and the fact that human society now produces chemicals that have never been produced by natural processes and to which all organisms are naïve, we know of no population-genetic or evolutionary processes that are unique to evolutionary toxicology. Thus, the methods and conceptual basis for studies in evolutionary toxicology can be derived directly from evolutionary biology and conservation biology (Bickham & Smolen 1994).

Basically, population genetic responses to xenobiotic stress fall into four categories: alterations in relative amount of genetic diversity; alterations in allele

or genotype frequencies as a result of contaminant-induced selection; alterations in gene flow and the genetic relationships among populations; and alterations in allele or genotype frequencies as a result of altered mutation rate (Theodorakis & Wirgin 2002, van Straalen & Timmermans 2002). Reductions in genetic diversity may occur via population crashes or bottlenecks, reduced reproductive output or recruitment (both from within and outside the population), or alterations in the relative birth and death rates. Anthropogenic reductions in genetic diversity are of concern because reduced genetic diversity may affect the growth, evolutionary plasticity, sustainability, and probability of extinction of populations. Also, reduced genetic diversity may lead to increased inbreeding and subsequent fixation of deleterious mutations. Furthermore, it has recently been argued that population genetic diversity may influence higher-level processes such as community structure and ecosystem function (Medina et al. 2007), so that pollutant-induced modifications in genetic diversity may affect these parameters as well (the effects of genetic diversity on community structure has been termed "community genetics" [Whitman et al. 2006]). In addition, polluted habitats are often highly modified, and habitat alteration/destruction and pollution may have additive or synergistic effects on population genetic diversity. For example, it has been found that individuals with lower genetic diversity are more susceptible to the effects of pollution than are individuals with greater genetic diversity (Nowak et al. 2007; Prus-Gowacki et al. 2006). Thus, habitat disturbance or resource exploitation affects on genetic diversity (Hoffmann & Daborn 2007; DiBattista 2008) could exacerbate the effects of environmental pollution on impacted populations. Although the "genetic erosion hypothesis" (van Straalen & Timmermans 2002) holds that pollution should reduce the level of genetic diversity, some studies have found that pollution may increase genetic diversity (Theodorakis and Shugart 1997; Baker et al. 2001; Cohen 2002; Matson et al. 2006; Theodorakis et al. 2006; Tsyusko et al. 2006). Possible explanations for the increase in genetic diversity include altered patterns of dispersal, increased rate of mutation or genomic rearrangements, or diversifying selection (Theodorakis and Shugart. 1997; Baker et al. 2001; Cohen 2002; Theodorakis et al. 2006; Tsyusko et al. 2006).

Alteration of gene or allele frequencies due to contaminant-induced selection may occur if individuals with certain genotypes are more susceptible than other individuals to contaminant exposure. Contaminant-induced selection may also affect the genetic diversity and evolutionary plasticity of populations, at least for loci that are under selective pressure and those that are linked to them. The ecological consequences and significance of genetic adaptations to environmental pollutants are discussed in more detail in Theodorakis and Shugart (1998).

Allele frequencies and genetic relationships may also be affected by pollution-induced changes in gene flow. Because gene flow is often important for maintaining sustainability of populations, particularly in fragmented habitats, alterations of gene flow may affect population persistence or sustainability (Theodorakis & Wirgin 2002). Patterns of genetic relatedness among populations may also be a consequence of alterations of dispersal or occurrence of extinction–recolonization or source–sink dynamics. Thus, patterns of interpopulation genetic diversity may be used as an indication that these events are

occurring (Theodorakis & Wirgin 2002). Finally, environmental contamination may serve as a dispersal barrier and lead to decreased gene flow and increased genetic isolation of contaminated populations. Such a situation has been found for plants living in heavy metal–contaminated soils (Mengoni et al. 2001).

Increased mutation rates may also affect genetic diversity of populations. Such mutations lead to increased genetic load in the population, which may affect average fitness of the population. An increased mutation rate is particularly important for small populations, in which increased genetic load may lead to decreased fitness, which would then lead to reduced population size, resulting in increased inbreeding and fixation of deleterious alleles, which would further reduce fitness, and so forth. Thus, such populations may spiral toward extinction in a process known as "mutational meltdown" (Gabriel & Bürger 1994).

Finally, population genetic responses can make important contributions to ecological risk assessments because alterations in population genetic structure or diversity may be sensitive or early-warning indicators of other effects, such as alterations of dispersal and recruitment, population growth or dynamics, loss of species, and changes in community structure (Cronin & Bickham 1998; Theodorakis & Shugart 1998). The study of the effects of pollution on genetic diversity and changes in population genetic structure due to bottlenecks, selection, or mutation is the focus of "evolutionary toxicology" (Bickham & Smolen 1994).

METHODS AND TECHNIQUES APPLICABLE TO EVOLUTIONARY TOXICOLOGY

Overview

There are numerous methods and techniques available to detect structural damage to DNA and other individual-level consequences that result upon exposure to environmental pollutants (Shugart et al. 1992; Shugart 1996, 1998). Genomic technologies are rapidly advancing and provide powerful tools to evaluate subsequent genetic consequences at the population level. The incorporation of these sophisticated technologies to measure change to genetic material extends our knowledge of contaminant effects beyond traditional parameters, such as reproductive and phenotypic effects to population-genetic and evolutionary effects (D'Surney et al. 2001; Rose & Anderson 2005; Leet & Richards, in press).

With respect to genotoxicants, application of appropriate methods and techniques of genetic ecotoxicology can document the cellular progression of genotoxic stress (Shugart 1996) from pollutant exposure that leads to damage of the organism's genetic apparatus, to change in gene expression and other types of irreversible somatic cellular damage like chromosomal damage. Connecting measurable, individual-level effects to eventual population-level consequences (Figure 13–1) is an important aim of evolutionary toxicology. Costs and effort will restrict experimental design, and these frequently result in the use of several methods of evaluation to support findings and gain the knowledge to draw conclusions. This restricted experimental design encourages a weight-of-evidence approach to establish a cause–effect relationship between events at the molecular

level and consequent changes at higher levels of biological organization (Walker et al. 2006).

There is a variety of genomic technologies and methods that can be used to generate molecular markers to evaluate a population's genetic structure. All have specific advantages and disadvantages that must be considered when developing a research strategy and drawing conclusions (D'Surney et al. 2001; Rose & Anderson 2005; Leet & Richards, in press). A brief overview is given for several techniques currently used to evaluate genetic diversity. Three methods selected evaluate polymorphism either by analyzing for protein polymorphism (i.e., several alternate forms [alleles] of proteins that relate directly to gene expression) using allozymes or DNA polymorphism (i.e., sequence differences at a particular site in the DNA usually as a result of nucleotide differences or variable numbers of repeated nucleotides) using randomly amplified polymorphic DNA (RAPD) or microsatellite methods. The final method uses microarray technology to address changes in gene expression that occur throughout the genome.

Allozymes

Allozymes are enzymes that vary in their electrophoretic mobility and are indicative of different alleles of single genetic loci. Allozyme genotyping can be an informative and rapid way to evaluate genetic diversity (Bickham et al. 2000). This technique relies on electrophoretic mobility in buffers at specific pH. Genetic diversity is apparent only if amino-acid substitutions lead to change in the net charge of the protein. Because these loci code for proteins with specific functions, ecotoxicological studies using these markers often find diversity that is associated with contaminant-induced selection (Theodorakis & Wirgin 2002). Because they are coding loci, however, selective constraints limit the amount of genetic diversity that can be maintained in populations. Thus, the technique has limited value for evaluating short-term and finer-scale genetic variation. Although they are considered an "older" technology, allozymes have been used in recent evolutionary toxicology studies (Laroche et al. 2002; Mulvey et al. 2002; Roark et al. 2005).

More sensitive proteomic technologies for evaluating protein expression throughout the genome are currently being developed (Bradley et al. 2002; Shrader et al. 2003), and these technologies may lead to identification of candidate loci for studies of selection in polluted populations. For example, Mosquera and colleagues (2003) used two-dimensional gel electrophoresis to examine genetic diversity in Mediterranean mussels (*Mytilus galloprovincialis*). Thirty-three polymorphic loci were found, based on electrophoretic mobility of alleles. Rather than relying on electrophoretic motilities, future studies could use high-throughput proteomic sequencing technologies, such as mass spectrometric techniques, to identify alleles based on amino-acid sequence or mass spectrum. Genetic analyses using amino-acid sequences would be more powerful than those based on identification of electrophoretic variants.

Microsatellites

Microsatellites are noncoding regions of DNA composed of arrays of repeating motifs of short nucleotide patterns. Differences in the number of repeat motifs in an array define microsatellite polymorphisms. Primers (less than twenty-five

nucleotides in length) to genomic DNA containing microsatellite loci are developed. Polymerase chain reaction (PCR) amplification produces base-pair products that are separated by gel electrophoresis. The microsatellite allele bands are analyzed using statistical programs to evaluate population-genetic structure. Genetic diversity is assessed on the basis of heterozygosity, allelic richness, and gene differentiation. Microsatellites are rapidly evolving loci that often display high amounts of genetic variation. Thus, microsatellite analysis is conducive to many situations, including field studies, where it may not be possible to collect large numbers of individuals as well as in situations where fine-scale discrimination of recently diverged populations are needed (Leet et al. 2007). If large numbers of microsatellite markers are available, they can also be used for "population genomic" techniques to identify markers linked to functional loci. Finally, because microsatellites are noncoding loci, they are not subject to selective constraints, so mutations are not quickly removed from the population. Because of their high mutability, microsatellites can be used to detect recent mutations. Microsatellite markers have been recently used to examine pollutant-mediated effects on genetic diversity in fish (Whitehead et al. 2003), mice (Berckmoes et al. 2005), mollusks (Piñeira et al. 2008), and plants (Tsyuko et al. 2006). They have also been used to examine mutation rates in swallows (Ellegren et al. 1997).

Dominant anonymous DNA markers

These types of markers typically produce "DNA fingerprint"–like banding patterns on agarose or polyacrylamide gels. Genetic diversity is then quantified on the basis of band presence or absence. Because "homozygous present" bands are indistinguishable from "heterozygous present/absent" bands, they are referred to as "dominant markers" (Theodorakis & Shugart 1998). The main advantage of such techniques is that they analyze large numbers of loci simultaneously and so often reveal high levels of genetic diversity. Two types of dominant markers that have been used in ecotoxicological studies are RAPD and amplified fragment length polymorphisms (AFLP). The following paragraph focuses on PCR-based analysis. Restriction fragment length polymorphism (RFLP)-based analysis of minisatellites has been used in a limited number of studies (e.g., Yauk et al. 2000) and is reviewed elsewhere (Theodorakis & Wirgin 2002).

Various methods have been developed to assay anonymous genetic markers that use PCR to amplify a large number of bands from which patterns or associations can be determined. The best of these is the AFLP method, which uses genomic DNA digested with various restriction enzymes, which are then ligated to short pieces of DNA of known sequence (adaptors). PCR primers that are complementary to the adaptors and the ends of the restriction fragment are then used to amplify these DNA fragments. PCR primers with three random bases at the 3′ end are used to reduce the number of amplified bands into a manageable number. These DNA fragments are then separated by polyacrylamide gel electrophoresis and identified by silver staining, stained with fluorescent DNA-binding dyes, or labeled with fluorescent probes. The process results in a banding pattern with typically 20–100 bands per reaction. The RAPD technique also employs PCR amplification of DNA sequences but uses a random set of short primers to generate typically ten to thirty bands per reaction. Problems of reproducibility stem

from different PCR conditions resulting in different band profiles and the fact that different laboratories typically produce different profiles. The AFLP technique is more labor-intensive than the related RAPD technique. The reproducibility of AFLP bands is greater, however; thus, this method is recommended. A third technique is inter-simple sequence repeat (ISSR) polymorphisms, which use microsatellite-based primers to amplify DNA sequences between closely spaced microsatellite loci (Pradeep et al. 2002).

The investigations of Theodorakis and Shugart (1999) and Theodorakis and colleagues (2006) demonstrate the utility of anonymous genetic markers as an effective tool to evaluate a population's genetic structure in environmental species. In particular, anonymous genetic markers are useful to screen for patterns among populations with different exposure histories and for identifying markers of selection. The latter is described in more detail in Box 13. The main advantage of the approach is that such markers have applicability to a wide variety of species. The main disadvantage is that rigorous standardization and quality controls must be adhered to in order to ensure reproducibility. Anonymous genetic markers have been used to examine population genetics in contaminated environments for plants (Muller et al. 2004), invertebrates (Ross et al. 2002; De Wolf et al. 2004; Piñeira et al. 2008; Gardeström et al. 2008; Martins et al. 2009), and fish (McMillan et al. 2006; Theodorakis et al. 2006; Williams & Oleksiak 2008).

Organellar DNA

Certain organelles – mitochondria and chloroplasts – contain their own DNA, which can be used for genetic analyses. Mitochondrial DNA (mtDNA) is a closed, circular molecule that exists in multiple copies within individual cells. It is almost always maternally inherited, does not undergo recombination, and contains thirteen protein-encoding genes, two ribosomal RNAs (rRNAs), and twenty-two transfer RNAs (tRNAs) with little intergenic space (Theodorakis & Wirgin 2002). Because mtDNA consists of highly conserved regions interspersed with regions of moderate-to-high genetic variability, it lends itself quite well to PCR-based DNA sequence analysis. The mitochondrial genome evolves at a much higher rate than genomic DNA and so provides a much finer-scale resolution than do genomic coding sequences (Theodorakis & Wirgin 2002). Because of its clonal nature of inheritance, mtDNA is also more sensitive to the effects of population bottlenecks than genomic DNA is. Because mtDNA is readily amenable to DNA sequencing, it can provide a large amount of genetic information, and it can be used in phylogeographic analysis. Recent evolutionary toxicology studies that have employed mtDNA include studies on rodents (Matson et al. 2000; Baker et al. 2001; Theodorakis et al. 2001), frogs (Matson et al. 2006), and invertebrates (Kim et al. 2003; Rocha-Olivares et al. 2004; Chung et al. 2008). Although, theoretically, all copies of mtDNA in a cell (or an organism) should be genetically identical clones, mutations in mtDNA may also lead to a condition known as heteroplasmy, a condition in which there are different mtDNA clones in a cell with different DNA sequences. Heteroplasmy can be used as a marker of increased mutation rates (Wickliffe et al. 2002; Matson et al. 2006), but it also may complicate analyses using mtDNA in population genetic studies.

Chloroplast DNA can also be used in genetic studies of plants in contaminated environments (Mengoni et al. 2001). Many of the same characteristics for mtDNA mentioned earlier in text can also be ascribed to chloroplast DNA. In addition, chloroplast DNA in plants may contain microsatellite sequences (Mengoni et al. 2001; Provan et al. 2001), which combine characteristics of both organelle DNA and nuclear microsatellites.

DNA sequencing and single nucleotide polymorphisms

Analyses of genomic DNA sequences employ some of the advantages of allozymes and mtDNA. The loci are usually of known function, so assessing significance of alterations in DNA sequence may be straightforward. Also, DNA sequences provide a lot of genetic information per locus and are amenable to phylogenetic analyses. Because of the diploid nature of these loci, however, DNA sequencing is not as straightforward. This difficulty can be overcome by using techniques such as single-strand conformational polymorphism to rapidly identify heterozygotes and homozygotes (Grompe 1993). Also, the amount of genetic diversity may be limited by functional and selective constraints. This limitation may be alleviated by examining genetic diversity in noncoding regions, such as introns, but not without sacrificing information on the functional significance of such variation. Analyses employing nuclear coding loci often use either loci with known significance to toxicological responses or fitness or loci with high variability. For example, Tanguy and colleagues (2002) examined genetic variation in the metallothionein gene in oyster (*Crassostrea gigas*) populations exposed to metals. Besides finding evidence of pollution-mediated effects on genetic diversity, tolerance to metals was also dependent on genotype in laboratory experiments and in the field (Tanguy et al. 2002).

In some situations, nuclear coding loci may be both highly variable and of functional significance. One example would be major histocompatibility complex (MHC) genes, which have a high amount of genetic variability. In fact, erosion of genetic variability in these genes has been associated with loss of immune function and increased parasite load (Allen 2008). These genes have been used in ecotoxicological studies in rodents (Pfau et al. 2001) and fish (Cohen et al. 2006).

Another tactic for using genomic loci is to use single nucleotide polymorphisms (SNPs). SNPs are point mutations that result in single base-pair divergence among DNA sequences (Brumfield et al. 2003). SNPs can be present in both coding and noncoding regions of the genome and thus can be used for analyses requiring neutral (e.g., estimates of migration or demographic parameters) and non-neutral markers (e.g., identifying signatures of selection). Because use of SNPs allows the simultaneous analysis of multiple loci and because of the fact that they are the most widespread class of sequence variation, they can provide much genetic information (Brumfield et al. 2003; Schlötterer 2004). SNPs can be identified by a wide variety of methods that are applicable to almost any species, so they can be readily applied to studies of evolutionary toxicology. SNPs are commonly analyzed using conventional methods such as DNA sequencing, allele-specific PCR, and single strand conformation polymorphism (SSCP; Brumfield et al. 2003), but alternative high-throughput methods such as GeneChip assays, mass

spectrometric analysis, and pyrosequencing can also be used (Syvänen 2001; see also Chapter 4 by DeWoody and colleagues). Although SNPs hold much promise for evolutionary toxicology, they have not yet been used for this purpose.

DNA microarrays

The incorporation of transcriptomics (the analysis of global gene expression) to evaluate changes in DNA sequences and expressed messenger RNA (mRNA) has greatly improved the detection of altered gene expression in organisms exposed to environmental pollutants (Snape et al. 2004). Nucleic acid microarray technology now enables the simultaneous screening of thousands of genes for differential expression among individuals and populations (Aardema & MacGregor 2002; Watanabe et al. 2007). Gene chips are prepared by printing on glass microscope slides PCR-amplified complementary DNA (cDNA) sequences for particular genes. Relative abundance of gene sequences or transcribed sequences can be compared between samples. Wullschleger and Weston from the Environmental Sciences Division of the Oak Ridge National Laboratory discuss in Box 13 a multistep procedure that incorporates analytical advances from the biomedical community with microarrays to determine up- and down-regulated genes for plants exposed to an environmental stress. Two potential applications of microsatellites in population genetic studies include 1) identification of candidate loci that might be under selection pressure from environmental contaminants, and 2) use as a tool for high-throughput genetic analysis.

Identification of candidate loci using microarrays involves identifying which loci are potentially responsible for tolerance mechanisms and determining if these loci are genetically variable a posteriori. There are two potential strategies that could be employed. First, gene-expression patterns in exposed and nonexposed individuals (either field collected or exposed in the laboratory) can be compared. Second, tolerant and resistant individuals can be obtained – either from polluted and nonpolluted sites or as a result of laboratory selection studies – and exposed to a pollutant. The gene-expression patterns in tolerant individuals could then be compared to those in sensitive individuals. In either case, differentially expressed genes could be cloned and sequenced for use in population genetic studies. Because use of microarrays requires known sequences for cDNA probes, such analyses may not be available for nonmodel organisms. Further information on using microarrays and other techniques for identifying candidate loci involved in adaptation to environmental variables can be found in Hoffmann and Willi (2008).

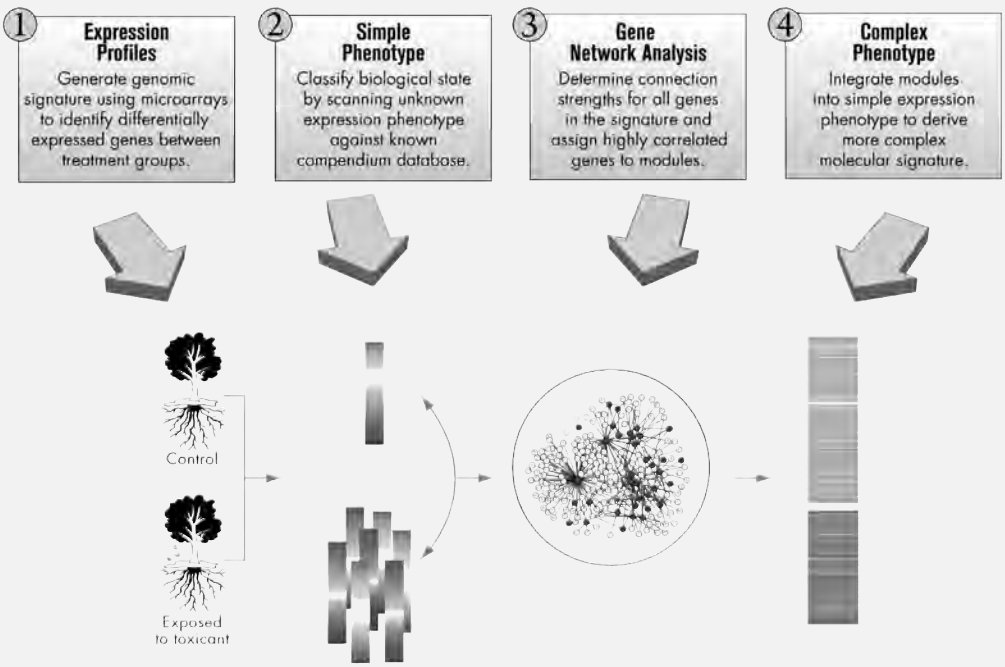
Microarrays can also be used as a high-throughput platform for determining DNA sequences (Chakravarti 1999). In this case, different alleles are immobilized on microarrays, and the array is hybridized to the genomic DNA of interest. Under stringent hybridization conditions, only those genomic DNA sequences that are completely homologous to the probes on the array will hybridize and provide a signal. By using this approach, genetic diversity in thousands of different loci may be monitored simultaneously (Chakravarti 1999). This technique, however, has yet to be widely applied in studies of population genetics, let alone evolutionary toxicology.

BOX 13: MICROARRAYS AND MOLECULAR PHENOTYPES

Stan D. Wullschleger and David J. Weston

Toxicogenomics is an emerging field of investigation that promises to add new insights to understanding how organisms respond to chemical contaminants or other stressors in aquatic and terrestrial environments (Snell et al. 2003; Klaper & Thomas 2004). Although the challenges likely to be encountered in incorporating genomics into ecotoxicological studies are many (Snape et al. 2004), a range of gene expression and profiling methods are available to help address the complex interactions of a species with its environment. Microarrays are such a technology (Gibson 2002) and provide a platform from which the genome-wide response of an organism to a given toxicant can be related to underlying genes and gene networks. Few groups, however, have tapped the full potential of microarrays especially as they relate to accelerating the discovery of toxicant pathways for biota in aquatic and terrestrial ecosystems.

Scientists from Oak Ridge National Laboratory are tackling this challenge by coupling microarrays with analytical advances from the biomedical community (Lamb et al. 2006). In a multistep procedure (Box Figure 13–1), microarrays are used to determine the up- and down-regulation of genes for plants exposed to a stressor (Step 1). The state of that organism is determined by scanning the observed fingerprint or phenotype against a compendium database that includes many expression signatures derived for plants exposed to a range of stressors. A novel, weighted gene coexpression network approach (Horvath et al. 2006)



Box Figure 13–1: Steps involved in creating a genomic signature using gene expression data from microarrays.

is then used to determine signaling networks and core genes underlying the expression phenotype (Step 3). This information is integrated with network properties to create a more complex and informative molecular signature (Step 4).

Weston and colleagues (2008) argue that such an approach, if successful and further verified, will allow the biologist to classify the molecular phenotype of an organism subjected to a given stressor and then link that to the genes and signaling pathways that govern the response. Microarrays used in this manner could provide a unique approach or early-warning indicator to the response of aquatic and terrestrial organisms to single- and perhaps multiple-chemical stressors in any environment. Although still early in the development phase, this signature-based approach coupled with network analysis could prove useful as a tool to interrogate the mechanisms that underlie complex biological responses to environmental stressors. It is likely that this concept will be applied in ecological genomics, stress physiology, ecotoxicology, and evolutionary biology. Thus, the use of genomic signatures or molecular phenotypes, as determined by microarray analysis, could serve as a new tool for scientific discovery.

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Which marker to use

Each of the markers just described has specific applications, limitations, and advantages (Schlötterer 2004). Therefore, the specific marker that should be chosen for population or evolutionary toxicology studies depends on the specific application. For example, if one is interested in analyzing for selective sweeps or signals of selection, then either coding loci known to be involved in the response to such toxicants or using a large number of markers (e.g., AFLPS) is advisable (Whitehead et al. 2003). Codominant markers such as microsatellites may be better for analyses that are based on allele frequencies (Whitehead et al. 2003). Highly mutable loci such as microsatellites and minisatellites (Ellegren et al. 1997; Yauk et al. 2000) might be used in both situations. Also, recent

research has found that RAPD markers may provide evidence of somatic mutations or other genetic rearrangements (De Wolf et al. 2004). Thus, RAPD markers may be well suited to examining effects of contaminants in general, but it raises an additional caution about interpreting differences in RAPD marker patterns on the basis of traditional population genetic theory alone. Also, even though allozyme analysis is often considered to be an "obsolete" technology, it consistently uncovers patterns of selection that might not be seen using other markers (Mulvey et al. 2002). In general, it should be kept in mind that any marker has not only its own advantages but its own pitfalls as well, and none are free from the possibility of genotyping errors. Such pitfalls should be thoroughly understood when interpreting data, and judicious methods of identifying and correcting for genotyping errors should also be employed (Bonin et al. 2004; Pompanon et al. 2005).

Assessing causality

Demonstrating population genetic effects of pollution may be problematic for a number of reasons. Such effects may occur as a result of alterations of population growth and dynamics due to acute or chronic toxicity, alterations in behavior, changes in community dynamics (e.g., competition, predatory/prey), or changes in productivity or trophic structure of ecosystems. Thus, changes in population genetic structure and diversity may be emergent properties of perturbed systems rather than direct effects of the toxicants themselves, so determination of the contributing factors to such perturbations may be difficult. One difficulty may be associated with the demography and dynamics of the populations themselves. For example, large effective population sizes or higher dispersal rates may mask population genetic effects of pollution (Pfau et al. 2001; McMillan et al. 2006). Several studies have also found a lack of genetic divergence among contaminated and reference populations, even when it was experimentally demonstrated that organisms from contaminated sites were more tolerant of pollution and that this tolerance was heritable (Muller et al. 2004; McMillan et al. 2006). An explanation for this finding could be 1) rapid evolution of tolerance without reduction of population size, or 2) a small number of loci involved in tolerance relative to the number of loci examined (Muller et al. 2004; McMillan et al. 2006). Conversely, it may appear as though there are contaminant-induced population genetic alterations when none are present due simply to stochastic fluctuations in genetic or demographic parameters. Also, there are many natural deterministic factors that may affect population genetic diversity (e.g., habitat structure, biotic or abiotic variables), irrespective of xenobiotic contamination. Consequently, separating natural from anthropogenic effectors of genetic diversity is an important consideration for population genetic monitoring. In addition, any two populations would be expected to differ in terms of genetic diversity and gene frequencies due to differences in evolutionary history and gene flow. Therefore, merely comparing a contaminated population with a reference population may not provide meaningful information about effects of contamination on population genetic structure.

A more robust approach would be to use a weight-of-evidence approach. The "criteria for assessing causality" outlined by Adams (2005) may be used but, because of the distinctive nature of evolutionary toxicology, some of these criteria may be directly applicable to determining population genetic effects of pollution, whereas others may require some modification. These seven causality criteria include 1) strength of association, 2) consistency of association, 3) specificity of association, 4) time order or temporality, 5) biological gradient, 6) experimental evidence, and 7) plausibility. It should be noted that it is often not possible to meet all of these criteria, but the strength of the causality argument increases with increasing number of criteria that are met. A description of how population genetic analyses can fit these criteria is given in the following section, and additional discussion can be found in Theodorakis and Shugart (1998), Theodorakis and Wirgin (2002), Baker and colleagues (2001), and Theodorakis (2003).

Strength of association

This criterion deals with whether the cause and effect coincide (Adams 2005) or if the endpoint in question is sensitive to pollutant stress. Because any two populations are expected to be genetically divergent, even if they reside in identical environments (due to different evolutionary histories), sampling from multiple reference sites and, if possible, multiple contaminated sites is required. Then, one way of demonstrating that cause and effect coincide or that genetic diversity is sensitive to pollutant stress would be to determine if the difference between the contaminated site(s) and any reference site is greater than what would be expected between any two reference sites. For example, a necessary criterion for establishing that pollution affects population genetic structure would be to determine if the amount of genetic diversity or frequency of a particular allele were lower at multiple polluted sites than at multiple reference sites. Examination of pollutant effects in multiple populations would minimize bias due to population-specific responses. Notable examples include studies that determined genetic variation in the estuarine fish *Fundulus heteroclitus* using allozyme (Mulvey et al. 2002; Roark et al. 2005) and AFLP analyses (McMillan et al. 2006).

Additionally, effects of contamination on population genetic structure could be inferred by determining if the observed patterns are different from what would be expected on the basis of evolutionary theory (e.g., expected genetic relationships based on inferred patterns of gene flow, common ancestry, or geographic distance and distribution). Therefore, careful choice of geographic locations of reference sites could allow testing to see if patterns of genetic diversity and relatedness among reference and polluted sites conform to those predicted by evolutionary theory. For example, Ross and colleagues (2002) examined population genetic diversity in crustaceans, and they chose reference sites that were both north and south of the contaminated site. This choice assured that differences among sites were not due to latitudinal gradients.

When determining if pollution affects population genetic structure, it is also advisable to pay close attention to possible genetic subdivisions or cryptic community diversity. For example, Woodward and colleagues (1996) examined heterozygosity in benthic chironomids and found that differences in heterozygosity

between polluted and reference sites were due to a heterogeneous distribution of allele frequencies within the population rather than pollution ("Wahlund effect"). Such an increase in genetic subdivision, however, could also be an effect of anthropogenic disturbance due to physical habitat disturbance or heterogeneously distributed contaminant concentrations. As an even more extreme example of how genetic differences can obfuscate pollution-induced effects, Rocha-Olivares and colleagues (2004) examined genetic diversity in marine harpacticoid copepods (a type of benthic crustacean). They found that pollution-induced changes in population genetic structure were due to the presence of two cryptic species (species that are superficially indistinguishable). In the polluted sites, one of the species was absent, which may appear as a change in the genetic diversity if it is assumed that the two species are part of the same intraspecific population.

In addition, differences in genetic diversity or genotype frequencies between contaminated and reference populations may be due to random factors (e.g., genetic drift, founder effects, stochastic disturbances) or neutral genetic variability (not affected by selection). To test for random effects, bootstrapping procedures (multiple subsampling with replacement) or other mathematical simulations could be used to test if the observed patterns of genetic diversity are statistically different from those generated by random sampling of genotypes. For example, Williams and Oleksiak (2008) used AFLPs to study population genetic structure in an estuarine fish, *F. heteroclitis*. They calculated F_{ST} values for each of the AFLP bands and compared these values to simulated distributions of F_{ST} values based on neutrality theory. They identified some markers that were different from expectations based on neutral theory and suggested that these "outliers" may be markers of loci that are under selection by the contaminants. To control for effects due to population history or environmental variables not related to pollution, they examined three separate populations affected by similar contaminants. A slightly different approach was applied by Timmermans and colleagues (2007). They used the ratio of silent to nonsilent mutations in DNA sequences to test the hypothesis of neutral evolution in the coding and promoter regions of the metallothionein gene in metal-exposed populations of the springtail *Orchesella cincta* (a type of soil arthropod). Cohen (2002) used a similar approach to examine MHC loci in polychlorinated biphenyl (PCB)- and PAH-contaminated populations of *F. heteroclitus* and found evidence of diversifying selection (resulting in an increased genetic diversity) in contaminated sites. Although rejection of the hypothesis of neutral evolution or nonstochastic distribution of genetic variation is a necessary line of evidence for inferring pollution-induced selection as a causal factor, it is not sufficient in and of itself to unequivocally demonstrate selection. The rejection of the hypothesis can be an important contribution, however, to multiple lines of evidence establishing strength of association and weight of evidence.

Consistency of association

Although demonstration of strength of association in population genetic studies is a necessary criterion for establishing causality, it is not sufficient alone. Another criterion would be demonstration of consistency of association: In other words, are the effects corroborated by other investigators and/or at other places or times,

or are similar effects seen in other species from the same locations? For population genetic studies, consistency of association may also apply to the consistent patterns seen when using multiple genetic techniques (e.g., concomitant responses for mtDNA, microsatellites, and allozymes) or multiple loci (e.g., multiple mitochondrial or microsatellite markers, multiple allozyme loci) and multiple types of analyses. For example, Theodorakis and Shugart (1997) and Theodorakis and colleagues (1998) found that RAPD markers that were elevated in frequency in radionuclide-contaminated populations of western mosquitofish (*Gambusia affinis*) in Tennessee were also elevated in frequency in radionuclide-contaminated populations of eastern mosquitofish (*G. holbrooki*) in South Carolina, and Southern blot experiments suggested that the DNA sequences of these loci were homologous. Tsyusko and colleagues (2006) also examined radionuclide-contaminated populations of two species of cattails (*Typha* spp.) and found similar increases in the amount of genetic diversity in both species. They cautioned, however, that their results also could have arisen due to factors such as mode of reproduction (sexual versus asexual) or to patterns of gene flow mediated by natural or anthropogenic factors not related to contamination. In another study, Whitehead and colleagues (2003) examined population structure of the Sacramento sucker (*Catostomus occidentalis*) using both AFLP and microsatellites and found concordant patterns among both markers. In another study, Piñeira and colleagues (2008), using a combination of AFLPs, microsatellites, and fitness-related markers, examined effects of an oil spill on the genetic diversity in mollusks.

It should also be noted that in some cases, different markers may not give the same answer due to the nature of the markers themselves. The same answers may be due to an advantage in evolutionary genetic studies (Whitehead et al. 2003). For example, rapidly evolving and conserved markers may be used to differentiate between patterns of recent genetic change and evolutionary history. Also, because mtDNA is maternally inherited and nuclear loci are biparentally inherited, comparisons of mitochondrial and nuclear genetic diversity may give an indication of sex-biased dispersal (Theodorakis et al. 2001).

Specificity of association

This criterion addresses the process of determining if the effect is diagnostic of exposure (Adams 2005) or, in other words, distinguishing stressor effects from environmental variability. The significance for population genetic studies is that there are environmental factors other than pollution that act as selective pressures or influence genetic diversity via modulation of dispersal, recruitment, population size, growth, and/or subdivision. One method of distinguishing between these natural and anthropogenic effectors would be to determine if patterns of population genetic diversity are concordant with indicators of exposure and effect, both at the individual and population level. Indicators of exposure include chemical body burden data and biomarkers of exposure, and indicators of effect include population- or community-level parameters (e.g., population declines), gross injury (e.g., malformations, fish or bird kills), or biomarkers of effect. For example, Keane and colleagues (2005) found that in populations of dandelion (*Taraxacum officinale*), tissue concentrations of heavy metals and particulate matter were correlated with levels of genetic diversity. Additionally, Benton and

colleagues (2002) found that population genetic diversity was correlated to average level of DNA damage in snail (*Pleurocera canaliculatum*) populations. In another study, Maes and colleagues (2005) found that genetic diversity was decreased in polluted populations of eels (*Anguilla anguilla L.*) and that individual levels of heterozygosity were negatively correlated to metal body burdens. Additional discussion of the integration of genetic biomarkers and genetic diversity in ecotoxicological studies can be found in Theodorakis (2001). Also, if contaminant-induced selection is suspected of contributing to patterns of frequencies of alleles or other genetic markers (i.e., individuals with a particular allele have a selective advantage in contaminated habitats), then the fitness components or biomarkers of deleterious effects should be genotype-dependent. For example, Laroche and colleagues (2002) found that in the flounder populations mentioned earlier in text, individual levels of DNA and fitness in individual fish were correlated to genotype and heterozygosity in a manner consistent with population genetic structuring. Furthermore, if contaminant-induced selection on particular alleles or genotypes is contributing to the observed differences in population genetic structure, then the association between biomarker response or fitness and genotype should be seen in contaminated populations but not in uncontaminated populations (such an association in the absence of contamination would indicate that this is a general phenomenon and not related to contamination). For example, Theodorakis and Shugart (1997) found that levels of DNA damage and fecundity were dependent on genotype in radionuclide-contaminated populations of *G. affinis*, but there was no such association in the noncontaminated populations.

Additionally, the experimental design should be such that the reference sites are as similar as possible to the study site and/or the environmental conditions of the reference sites bracket those of the study site. For example, if the contaminated site is a small stream with a silt substrate, then similar streams could be chosen for reference sites. There would still be some environmental variation even between the most similar of sites, so in another tact, the reference sites could be chosen to represent a distribution of environmental conditions. It could then be determined if the patterns of genetic diversity of contaminated sites fall within or outside of this distribution. These analyses should also examine the relationship between geographic distance or spatial structuring and genetic distance or population structure using tests such as Mantel tests or spatial autocorrelation. Such tests can be used to differentiate between genetic relationships mediated by contaminants and those due to geographic distance or distribution. For example, Mulvey and colleagues (2002) found that there was no correlation between geographic distance and genetic distance among populations of mummichog, but Mantel tests showed that there was a correlation between genetic distance and levels of contaminants in the sediments. Natural biogeography should also be taken into account when trying to determine if there are any effects of pollution on population genetics. For instance, in the Whitehead and colleagues (2003) study mentioned earlier in text, it was found that biogeographical factors such as location within a drainage, location of the drainage basin, and watershed geography described the data better than historical pesticide exposure did. Using analysis of molecular variance (AMOVA), they found that watershed geography, rather

than contamination history, accounted for much of the partitioning of genetic variation among sites. Similarly, Tsyusko and colleagues (2006) found that latitude, geography, and watershed location had a small but significant impact on microsatellite diversity in cattails near Chernobyl, Ukraine. To examine such relationships in the future, one promising approach may be to incorporate landscape genetic analyses into evolutionary toxicological studies. Landscape genetics analyzes spatial genetic data without the requirement of identifying discrete populations in advance (Manel et al. 2003). As the name implies, it involves spatially explicit interpretations of patterns of genetic variation and gene flow in the context of landscape structure (Manel et al. 2003; Storfer et al. 2006). This could involve integrating spatial analyses and Geographic Information System (GIS) and remote sensing technologies into population genetic and ecotoxicological studies (Storfer et al. 2006).

Examination of the environmental effectors of population genetic structure could also contribute to differentiation between natural and contaminant effects on populations. For example, multivariate techniques could be used to discern which environmental variables contribute the most to genetic differences among reference sites, and then it could be determined if the difference between contaminated and reference sites is different than what would be expected based on these confounding variables. Or, environmental contamination could be included as one of the environmental variables, and multivariate statistics could be used to determine if contamination was one of the major contributors to genetic differences between populations. For example, Timmermans and colleagues (2007), Janssens and coworkers (2007, 2008), and Laroche and colleagues (2002) used multivariate techniques such as principle component analysis and redundancy analysis to determine correlations among contaminant concentrations, fitness parameters, and genotype. These techniques can also be used to test the relationship between genotype and other environmental variables that might affect genetic diversity irrespective of the level of contamination.

Finally, to determine specificity of association, population genetic analyses might benefit from integration with population demographic studies, which might include mark-recapture or grid-trapping studies to obtain estimates of parameters such as population size, sex ratios, age structure, and patterns of dispersal and movement (Matson et al. 2000; Baker et al. 2001). Such studies may also be used to differentiate between alternative hypotheses used to explain differences in genetic diversity, such as changes in mutation or migration rates (Matson et al. 2000; Baker et al. 2001).

Time order or temporality

This criterion implies that the assumed stressor precedes the observed effect and that the pollution effect must diminish when the stressor is remediated. Because it may take many generations for population genetic effects to become manifest, demonstration of this criterion may be difficult for long-lived species. Reversal of effects of pollution is also dependent on gene flow. For species with shorter generation times and higher levels of gene flow, reversal of population genetic effects may be relatively rapid. Reversal would be particularly true for selection-mediated changes in population genetic structure if individuals with resistant

genotypes were at a selective disadvantage in noncontaminated environments. In addition, in some cases, it may be possible to monitor differences in population genetic structure before and after a specific event – for example, a chemical or oil spill (Piñeira et al. 2008) or construction of a factory or power plant. Monitoring would be possible only if prior information on genetic diversity is known. If not, then genetic changes after the event can be monitored over time and compared to temporal patterns of nonimpacted populations.

Population genetic effects are distinctive ecotoxicological end points in that the history of the population is reflected in current patterns of genetic diversity. The patterns may contribute to determining whether the stressors preceded population genetic effects. For one thing, different measurements of genetic variability may be able to distinguish between recent and historical effects. For example, number of alleles is more sensitive to recent events than is average heterozygosity. There are newer tests of recent population bottlenecks, and some of them rely on calculating the observed levels of heterozygosity and comparing them to those expected under models that assume mutation/drift equilibrium (Berckmoes et al. 2005). Also, certain markers (e.g., microsatellites) may evolve more quickly than others (e.g., allozymes) or may be more sensitive to the effects of bottlenecks (e.g., mtDNA). Differences between polluted and reference sites being revealed by number of alleles or haplotypes for microsatellites and mtDNA, but not for average heterozygosity, haplotype diversity, or by allozyme analysis, would suggest that the differentiation between contaminated and reference sites has occurred relatively recently.

Phylogenetic relationships may also be a way of discriminating between recent and historical influences on genetic diversity (Avise 1998). The discipline of phylogenetics examines evolutionary relationships between populations or between alleles/haplotypes, which are usually represented visually by a phylogenetic tree. Phylogenetic relationships between populations would be important in discerning patterns of gene flow, as well as in experimental design. For example, in choosing reference sites, it would be best not to develop a sampling scheme where a contaminated site is in one clade (i.e., a branch on a phylogenetic tree representing a group of evolutionarily related individuals) and all reference sites are in a different clade. Otherwise, differences between contaminated and reference sites may be due to evolutionary history rather than contamination. It would be better to either select reference sites that are in the same clade as the contaminated site or choose reference and contaminated sites that are homogeneously distributed among clades. Also, the topology of the tree may provide information as to the relative age of alleles or haplotypes: The younger haplotypes or alleles should be distributed among the terminal branches, whereas the older ones would be more deeply "rooted" in the tree, and this information can be used to infer mutational history. The topology of the dendrogram may also give information about demographic parameters; for example, a tree with long terminal branches may indicate recent population expansion or growth or the direction of dispersal among populations (Nielsen & Beaumont 2009).

A sub-branch of phylogenetics is termed "phylogeography," which integrates evolutionary relationships among alleles or haplotypes with their biogeographical distributions. This integration might provide information as to the effects

of geography on genetic relationships among populations, which can be used to determine if the pollution has caused the effects. There are statistical phylogenetic approaches: For example, a technique known as nested clade analysis (Templeton 2008) can be used to distinguish between effectors of gene flow and evolutionary history on population genetic structure. This technique has been used by Kim and colleagues (2003), for example, to infer that heavy-metal pollution has restricted gene flow. There has been some recent controversy over this technique, however: Some have argued that the analysis is inherently and fatally flawed (Petit 2008), whereas others have defended the reproducibility of the analysis given proper experimental design and rigor of analysis (Garrick et al. 2008; Templeton 2008). Nonetheless, there are also other statistical tests that can be used for analysis of phylogenetics, and these tests can be applied in a geographic context (Knowles 2009; Knowles & Maddison 2002; Templeton 2004; 2009; Nielsen & Beaumont 2009).

Biological gradient

This criterion asserts that a gradient in pollution should correspond to a gradient in genetic diversity and genotype frequencies. Data from biological gradients can be gathered by sampling sites that are various distances from a known source of contamination or by sampling various locations that are known to have differing levels of contamination. In one such study, Yauk and colleagues (2000) used microsatellites to calculate mutation rates in herring gulls, and they found that the mutation rate decreased with increasing distance from the source. In addition, Berckmoes and colleagues (2005) found that genetic distance among populations of mice increased with increasing distance from a metal ore smelter, but the experimental design precluded distinguishing between effects of contamination and other factors. DeWolf and colleagues (2004) also found that population genetic diversity varied in concordance with a gradient in metals in a marine gastropod. We should be cautioned, however, that there may be other gradients that affect genetic diversity (e.g., temperature, soil chemistry) that may covary independent of pollution exposure. For instance, genetic diversity may increase in a downstream trend in creeks and rivers regardless of contamination. Because contaminant concentrations increase downstream of a discharge pipe (due to dilution, sequestration, and degradation of contaminants), there may be a correlation between genetic diversity and contaminant concentrations even though there is no mechanistic connection. In addition, wastewater effluents may alter the environment in ways that have nothing to do with contamination (e.g., by increasing water flow in a small stream), and this alteration may indirectly affect genetic diversity. With judicious experimental design and careful analysis of data, however, such effects can be factored into the interpretation of the data.

Experimental evidence

Experimental evidence for evolutionary toxicological effects may include laboratory exposures, in situ caging studies, or microcosms/mesocosms. To establish evidence of causality, similar responses should be seen for field and laboratory exposed populations. For example, Gardeström and coworkers (2008) exposed

laboratory populations of copepod crustaceans to copper-spiked sediment. Using AFLP markers, they found that exposed populations not only showed reduced genetic diversity but also increased interpopulation differentiation (increased F_{ST} values). They did not find, however, concordant decreases in abundance. They therefore concluded that the observed effects on diversity and F_{ST} were due to selection for resistant genotypes, rather than to genetic bottlenecks or drift (Gardeström et al. 2008). This hypothesis could be further tested by using coalescence-based simulations of null distributions of F_{ST} values discussed earlier in text (Williams & Oleksiak 2008), by using breeding studies to examine heritability of resistance, or by determining if fitness components were genotype-dependent.

In general, genotype dependence of fitness components (e.g., survival, reproduction) can be demonstrated in experimental studies. For example, it has been found that survival in mercury-exposed mosquitofish (*Gambusia holbrooki*) in the laboratory was correlated with allozyme genotype and that this correlation was relatable to population genetic patterns in contaminated populations in the field (Heagler et al. 1993). In subsequent work, Tatara and coworkers (2002) found an increased frequency of the resistant genotypes in microcosm populations of mercury-exposed mosquitofish that was stable over multiple generations. Ross and colleagues (2002) examined genetic diversity in metal-contaminated populations of isopods and prawns and found contamination-associated patterns of genetic diversity in the isopods but not the prawns. Experimental laboratory exposures found that mortality in metal-exposed prawns was not genotype-dependent, but it was for metal-exposed isopods. Another experiment focusing on microcrustaceans was reported by Martins and coworkers (2009). They collected *Daphnia longispina* from acid mine drainage–contaminated and reference populations and cultured them in the laboratory. They then exposed the laboratory populations to metals and recorded average time to death (TTD). Using the Mantel test, they found statistically significant correlations between genetic distance and differences in TTD among populations. They also found that TTD was associated with genotype within populations. All of the studies just mentioned are significant because they provide supporting evidence for patterns of genetic diversity seen in the field.

An alternative experiment would be to artificially select for tolerant and resistant phenotypes and then determine if differences in genotype correspond to differences seen between contaminated and reference sites in the field. Such an approach was attempted by Roark and coworkers (2005) to determine if laboratory selection of *F. heteroclitus* exposed to PCBs would result in shifts in allele frequencies similar to those seen in PCB-contaminated field populations, but the results were equivocal. In a related approach, Theodorakis and Shugart (1997) examined *G. affinis* from two populations: a radionuclide-contaminated population (Pond 3513) that was founded by intentional introduction of individuals from a reference population (Crystal Springs) in 1977 and that reference population. They collected individuals from Crystal Springs and caged them in Pond 3513. Not only did they find that survival and levels of DNA damage were genotype-dependent in the caged individuals, they also found that the genetic distance between the survivors and the indigenous Pond 3513 population was smaller

than that between the survivors and the original Crystal Springs population. Crystal Springs fish caged in a noncontaminated pond, however, were still more similar to the original Crystal Springs population than to the population in the pond in which they were caged.

Plausibility

This criterion requires a biologically plausible mechanism whereby the stressors can induce the effects. In an evolutionary toxicological context, this would require that contaminants exist (or have existed in the past) at levels great enough to affect fitness components (growth, survival, reproductive success, embryo/larval development), recruitment, mutation rates, or gene flow. Studies that demonstrate that a contaminant causes effects on population dynamics or demographic parameters could also be used to fulfill this criterion. If there is a hypothesis that the data indicate that there is selection for certain alleles or genotypes, there must be a mechanism for such selection that is consistent with previous findings or theoretical principles. For example, Roark and colleagues (2001) found that allozyme heterozygosity was correlated with levels of hypoxia in mosquitofish in an urban river. Because these allozymes were involved in aerobic respiration, and such loci have been associated with allele-specific differences in respiration (Tatara et al. 2001), a mechanism of adaptation to hypoxia is plausible.

In another notable example, Timmermans and coworkers (2007) and Janssens and colleagues (2007, 2008) examined the DNA sequence of the metallothionein coding and promoter regions in the springtail *O. cincta*. They found that allele frequencies and patterns of genetic diversity differed between metal-contaminated and reference populations. They found one promoter allele in particular, allele "pmtD2" that was elevated in contaminated sites, and multivariate analyses indicated that the frequency of this allele was most strongly associated with environmental cadmium concentrations. Tajima's D test, which is based on the ratio of silent to nonsilent mutations, rejected the hypothesis of neutral variation at this locus. In subsequent studies, Janssens and coworkers (2007) cloned the various promoter alleles into luciferase expression vectors and examined the levels of cadmium-induced expression in vitro. They found that the expression levels mediated by the pmtD2 allele were much higher than those mediated by many other alleles. These findings are consistent with results that showed that heavy-metal tolerance in invertebrates is associated with levels of metallothionein expression and that levels of cadmium-induced metallothionein expression is an additive genetic trait (i.e., subject to selection; reviewed in Timmermans et al. 2007).

The previous studies focused on noncoding *cis*-regulatory elements of genes, but genetic variation may occur in the coding portion of the genes as well. In such an instance, the plausibility criterion would require demonstration that genetic variation results in nonsilent substitutions in amino acid sequence, that such modifications occur within the functional regions of the molecule, and that the variation has ramifications for health or fitness parameters. For example, Cohen (2002) and Cohen and coworkers (2006) found that genetic diversity

of MHC alleles in *F. heteroclitus* was apparently affected by environmental contamination. They found evidence of increased genetic diversity, possibly due to diversifying selection. These findings were also concordant with a contaminant-induced alteration in parasite communities (a possible selection pressure) and with the finding that MHC diversity was correlated to parasite load (a possible link to fitness). DNA sequencing of these alleles found that many of the variable locations were in functional regions of the protein, such as antigen-binding sites (Cohen et al. 2006). Continuing work is pursuing evidence of relative immune function among the genotypes (Cohen et al. 2006).

CASE STUDIES

Azerbaijan

Since 1995, studies have been conducted on the effects of pollution on fish and wildlife populations in Azerbaijan, with special interest in the city of Sumgayit. Azerbaijan sits in the midst of the hot spot of Caucuses biodiversity, which is one of the twenty-five richest and most threatened areas on the planet (Myers et al. 2000). The country faces difficult challenges as it develops its post–Soviet era economy. In particular, impacts resulting from poor environmental safety practices during the Soviet era are severe in Baku, Sumgayit, and in many oil fields around the country. The situation in Sumgayit is particularly grave, in that contamination of the environment from multiple industrial sources and the wastewater treatment plant is extensive and threatens the health of city residents. In fact, Sumgayit was recently recognized as one of the world's ten most contaminated cities by the Blacksmith Institute. To make matters worse, many refugees from the occupied areas of western Azerbaijan now live in Sumgayit, including in its industrial zone. These people are particularly at risk due to their living conditions and daily activities.

Wildlife in and around Sumgayit show the effects of chemical exposures. Biomarker studies were conducted of turtles and frogs from the contaminated wetlands (Figs. 13–2 and 13–3) scattered around the city compared to animals collected at more pristine sites located in the mountains and elsewhere around the country (Matson et al. 2005a,b). Within Sumgayit are several sites with high levels of pollutants of various kinds. Mercury is a widespread contaminant in Sumgayit. It has been found at high levels in ponds along the Caspian Sea coast such as the one adjacent to the wastewater treatment plant, as well as near the chlor-alkali plant (the site where an estimated 1,566 tons of mercury have been spilled; Bickham et al. 2003). PAHs are commonly found in oil or produced as by-products of combustion. Some PAHs are highly toxic and, like mercury, are mutagenic. PAHs are high in certain ponds, including the ones near the wastewater treatment plant, as are other potentially mutagenic chemicals like PCBs and many organochlorine pesticides like lindane and DDT. Residues of these chemicals have been found in sediments and in the tissues of turtles and frogs (Swartz et al. 2003; Matson et al. 2005a,b). The presence of such a complex mixture of

Figure 13–2: Two panoramic views of the wetlands adjacent to the industrial wastewater treatment plant in Sumgayit, Azerbaijan (photos courtesy of Cole Matson). In the top panorama (a), the Caspian Sea can be seen in the background.

Figure 13–3: The ponds adjacent to the industrial wastewater treatment plant in Sumgayit, Azerbaijan, are home to a variety of fish and wildlife species that are the focus of ecotoxicological studies (photos courtesy of Cole Matson). (A) shows two species of freshwater turtles including the Caspian turtle (*Mauremys caspica*, closest in the foreground) and European pond turtles (*Emys orbicularis*, the remaining turtles). (B) shows a dice snake (*Natrix tessellata*) consuming a mosquitofish (*Gambusia* sp.). The mosquitofish is an invasive introduced to Europe from the United States. (C) shows the marsh frog (*Rana ridibunda*).

contaminants in the environment and in animal tissues is cause for great concern but, as discussed earlier in text, it does not by itself confirm damage to the wildlife populations.

Biomarker analyses were conducted to determine if the chemical exposures are affecting wildlife. These studies were designed to detect genetic damage in somatic cells of turtles and frogs and, if such damage were present, to identify which contaminant or contaminants were the cause of the damage. To do this, two methods were used to detect genetic damage in blood cells. The micronucleus assay is commonly used as a biomarker of genetic damage; our collaborator, Dr. Grigoriy Palatnikov of the Karaev Institute of Physiology in Baku, has examined blood smears from hundreds of turtles, frogs, and other animals during the course of these studies. Micronuclei form in blood cells as a result of chromosomal breaks or aneuploidy produced by exposures to mutagens. We also use a procedure called flow cytometry (FCM), which is a more automated analysis. Cell-to-cell variation in DNA content results from chromosomal breaks and rearrangements caused by exposure to chemical contaminants (Bickham et al. 1992, 1994). Both the micronucleus and FCM tests are capable of detecting genetic damage in wildlife and fish and have proven to be sensitive biomarkers to the effects of a variety of chemical mutagens and radioactivity (Bickham 1990, 1994). Increases in the frequencies of micronuclei or in the coefficient of variation of cellular DNA content are indicators of genotoxic exposure and effect.

Studies on turtles and frogs showed that micronuclei and FCM tests revealed genetic damage in blood cells from animals taken at the wastewater treatment plant ponds, as well as ponds adjacent to the chlor-alkali plant, compared to matched reference sites. Correlation analyses showed mercury and PAHs to be the likely causes of the observed genetic damage (Swartz et al. 2003; Matson et al. 2005a,b).

Population genetic analyses of frogs were conducted using nucleotide sequences of the mtDNA control region (Matson et al. 2006). Specifically, nucleotide sequences were examined from frogs from Sumgayit, Ali Bairamly, and Alti Agach. The latter two sites served as reference or pristine sites because frogs from those areas did not show evidence of genotoxic damage using FCM or micronuclei.

The mtDNA is a small piece of DNA located outside the cell nucleus. It shows strict maternal inheritance, and typically an individual has only one form of mtDNA. Using the mtDNA sequences, it was shown that frogs from Sumgayit have lower genetic diversity, including both haplotype and nucleotide diversity, than do frogs from the reference areas. A closer examination of the patterns of distribution of the different forms of mtDNA revealed that gene flow, or the effective migration of female frogs from one site to another and subsequent reproduction, was predominantly into the Sumgayit region. Therefore, Sumgayit has acted as an ecological sink, which is indicative of a long-term problem with successful reproduction. That is, excess reproductive output from surrounding pristine areas provides an input of migrants into Sumgayit, and the resident frogs in Sumgayit have relatively poor reproductive success or survival. Thus, the genetics data revealed an important ecological effect of the chemical exposures.

In addition, evidence was found of an increased mutation rate of the mtDNA at the most contaminated site in Sumgayit. The ponds adjacent to the wastewater treatment plant had the highest levels of haplotype and nucleotide diversity among the sites examined within Sumgayit. Moreover, a few individuals were observed that possessed two forms of mtDNA. These "heteroplasmic" frogs show the initial stages of new mutations, and it is assumed that in a few generations the new mutations will become "fixed" in their offspring. The observation of heteroplasmy is rare in nature. Matson and colleagues (2006) examined a total of 207 marsh frogs. Of that total, only seven frogs were observed to be heteroplasmic. Of these, six were from the wastewater treatment plant ponds and one was from an adjacent area. Frogs from other ponds in Sumgayit or from the reference areas showed no evidence of heteroplasmy.

New mutations initially occur in the heteroplasmic state but become fixed due to a bottleneck in the population of mitochondria that occurs during oogenesis in each generation (Pakendorf & Stoneking 2005). Thus, heteroplasmy is typically an ephemeral characteristic and is not expected to last long within a population. It is usually the association of a common haplotype with a rare haplotype; the rare haplotype is inferred to be the new mutation. New mutations are expected to be rare (depending on the population size), to have limited distributions because they have not existed for sufficient time to be broadly distributed, and potentially to exist in the heteroplasmic state. In studies of evolutionary toxicology, it is desirable to differentiate among naturally occurring and pollution-induced mutations. Although a new mutation can never be directly observed to result from a contaminant, the co-occurrence of new mutations with contaminants is strong evidence of cause and effect. In the case of the marsh frogs of Sumgayit, it is precisely the occurrence of heteroplasmy, unique rare alleles, and the subsequent higher diversity estimates for the most contaminated sites that supports the conclusion that these are contaminant-induced mutations.

Considering all of the findings just summarized, it is apparent that wildlife from the Sumgayit industrial zone is experiencing genetic damage both at the somatic level (i.e., cells from the body of the animal) as well as at the population level. In this series of studies, the presence of potentially genotoxic and nongenotoxic chemicals was documented in sediment samples and tissue samples of frogs and turtles. Biomarker studies confirmed somatic chromosomal damage, and correlation analyses indicated that it was likely due to mercury and PAHs, both of which are known genotoxins. On a regional scale, population genetic studies showed that effective female migration primarily into Sumgayit is potentially the result of reduced reproductive output and evidence of an ecological sink. Overall reduced genetic variability for the Sumgayit region is interpreted as the result of an historic bottleneck with subsequent diversity loss due to genetic drift that likely occurred as a result of the construction of a large number of chemical plants beginning in the 1940s. At a finer geographic scale, the presence of heteroplasmic individuals and higher diversity estimates at the most contaminated site within Sumgayit is evidence of induced mutations and thus an increase in the mutation rate. This study illustrates the necessary connections to be made among exposure, biomarker effects, and emergent population genetic effects that are at the core of evolutionary toxicology.

Pigeon River

The Pigeon River is located in North Carolina and Tennessee and drains into the Tennessee River. Since 1908, the Pigeon River has been severely impacted by paper-mill discharges. Since 1988, biomonitoring studies have found that redbreast sunfish (*Lepomis auritis*) have exhibited a reduction in fitness parameters, health indices, and population density. In addition, there has been evidence that fish populations at the contaminated sites may have been ecological sinks and may have been largely sustained by immigration from neighboring nonimpacted streams. The flow of water in this river has been diverted in one section so that the Pigeon River kilometer (PRK) 42 site no longer receives contaminated flow from upstream. Hence, the biomarker and bioindicator analyses (molecular, biochemical, physiological, and population- and community-level end points) measured at these sites indicated that the degree of contaminant impact decreased in the order of PRK 89 > PRK 103 (upstream of the mill) > PRK 27 > PRK 42 Little River > Little Pigeon River. Thus, the biomarker data suggest (at least partial) recovery at site PRK 42 subsequent to diversion of the main river channel. Effects of this contamination on population genetic structure were not examined, however. To this end, DNA was analyzed using RAPD. Genetic analyses included genetic distance between populations, levels of gene flow among populations, and genetic relatedness among the individual fish. It was found that the level of genetic diversity was higher in the Pigeon River populations than in the reference populations. Patterns of genetic distances among populations may have been impacted by the pulp-mill contaminants because they could not be completely explained by drainage patterns. Phylogeographic analysis, maximum likelihood (MLE) analysis, and assignment tests indicated a higher immigrant/emigrant ratio in the contaminated sites, which suggests that the contaminated populations may be more "sinklike." This finding is consistent with the hypothesis that pulp-mill contamination lowers the population density of the affected redbreast sunfish populations because populations in which the density is much lower than the carrying capacity would produce fewer emigrants, and any immigrants would be more likely to become established than in high-density, nonimpacted populations. A sinklike population may experience higher genetic diversity if it receives genetic input from many different populations (thus farther from migration–drift equilibrium than other populations). Finally, a cladistic analysis using neighbor-joining and minimum-spanning trees, and MLE analysis, suggested an elevated mutation rate in the more impacted sites. This finding is consistent with previous research that found an elevated level of DNA strand breakage in the liver and a higher mutagenic potential (using the Ames assay) in muscle extracts in these fish. Thus, the higher genetic diversity in the Pigeon River populations may have been affected by altered gene flow and mutational processes as a result of pulp-mill effluent discharge.

This study illustrates the ways in which multiple lines of evidence can be used to infer causality between chemical exposure and population genetic effects. Regarding the strength of association criterion, this study used multiple reference sites and multiple contaminated sites. The consistency-of-association criterion was met because similar results were obtained with different analyses. Also, the

specificity-of-association criterion was met because the patterns of genetic diversity could not be explained by geography alone. The time-order/temporality criterion was met when a change in the river channel diverted contaminated water away from a previously contaminated stretch of river. The patterns of genetic diversity in this site were more similar to the reference site than to the contaminated sites in the same river, indicating possible recovery. In addition, the biological gradient criterion was met because sites closer to the paper mill were more divergent – in terms of patterns of genetic diversity – from reference sites than were the downstream sites. Thus, a gradient of biological responses ranging from biochemical biomarker to community-level effects was observed. The plausibility criterion was met because previous studies showed that the levels of contaminants in this river system were high enough to affect fitness components and population demography, and extracts from these sunfish were found to be mutagenic in vitro (Theodorakis et al. 2006).

STATISTICAL METHODS FOR EVALUATING POPULATION GENETIC STRUCTURE

There are a number of ways in which genetic data can be analyzed. Many early studies used traditional analyses, such as analyses of genetic diversity, population fixation indices, or Mantel tests. A number of approaches have been developed in the last ten to fifteen years, however, that have been getting increased usage in recent years. These include AMOVA, multivariate analyses, coalescence-based approaches, MLE analyses, and Bayesian analyses.

Although it was initially published in 1992 (Excoffier et al. 1992), the AMOVA was not widely applied in evolutionary toxicology until after the turn of the twenty-first century. This analysis is analogous to the parametric analysis of variance tests (Excoffier et al. 1992). AMOVA can test the proportion of overall genetic variation attributable to variation within populations, between populations, and between groups of populations in a region. It can also be used to determine the relative partitioning of genetic variability among groups (e.g., contaminated versus reference sites), between populations within groups, or within populations. In evolutionary toxicology studies, this analysis has been applied to rodents (Theodorakis et al. 2001; Berckmoes et al. 2005), fish (Whitehead et al. 2003; Theodorakis et al. 2006), springtails (Timmermans et al. 2007), and *Daphnia* (Martins et al. 2009).

Another type of analysis that is receiving more attention is multivariate analysis of genetic data. Techniques such as nonmetric multidimensional scaling principal component analysis and redundancy analysis have recently been used in evolutionary toxicology studies (Laroche et al. 2002; Theodorakis et al. 2006; Timmermans et al. 2007). Such analyses can be used to examine genetic relationships among populations without forcing them into a bifurcated tree and to examine relationships between allele frequencies and contaminant concentrations or other environmental variables.

Recently, the use of coalescent theory has also gained increased attention in population genetic studies of contaminated sites (Rosenberg & Nordborg 2002).

Coalescence is a stochastic model that uses simulated genealogies to make inferences regarding the evolutionary processes that shape observed patterns of polymorphism and genetic diversity, both within and between populations. Coalescent models are not used to construct phylogenies but rather estimate parameters such as recombination, migration, selection, and so forth that may give rise to the genealogical patterns. The coalescent is commonly used as a simulation tool to test hypotheses, for example, by simulating a distribution of data sets under a null hypothesis (e.g., no mutation, selection, migration) and determining how frequently the observed patterns of polymorphism in an actual data set are simulated. Coalescence theory is also the basis for "population genomic" analyses (Luikart et al. 2003). This method was used with *F. heteroclitus* from Superfund sites in the eastern United States (McMillan et al. 2006; Williams & Oleksiak 2008). Analysis of multiple loci, such as AFLPs or large numbers of microsatellite loci, is used to identify loci that show interpopulation relationships that significantly deviate from those expected based on neutral evolution (Luikart et al. 2003). Finally, the simulated distribution of genealogies can be used to calculate confidence intervals for various population parameters, such as heterozygosity and migration rates. The coalescent is often used as a basis for MLE and Bayesian analyses.

MLE analyses are those that use a fixed set of data and underlying probability model to choose the values of the model parameters that make the data "more likely" than any other values (Holder & Lewis 2003). MLE can, for example, be used to estimate population parameters such as mutation and migration events, given a set of genetic data and a particular model (i.e., an equation that describes a particular distribution; Beerli & Felsenstein 2001). MLE will then randomly choose values for the model parameters (e.g., "constants" of an equation) until values are found that produce a model that best fits the data. MLE can also be used to construct phylogenies or "trees." In this case, all possible trees are constructed, and the likelihood that each tree explains the data is calculated. These likelihoods are based on the chance that different genotypes would have a common ancestor. This chance can be calculated on the basis of the number of different bases in the two sequences, the rate of mutation from one base to another, and the type of mutation required to produce two different sequences (e.g., transitions from one purine to another are more likely than transversion mutations between purines and pyrimidines). As one would expect, MLE procedures can be quite computationally intensive and, even with advanced computer programs, such calculations may take days or even weeks to calculate. In evolutionary toxicology studies, a Markov Chain Monte Carlo MLE approach was used to estimate asymmetric patterns of gene flow between contaminated and reference populations of fish (Theodorakis et al. 2001).

Bayesian analysis is similar to MLE analysis in that it employs a likelihood function, which in Bayesian analyses is a conditional distribution that stipulates the probability for the observed data given a particular value of the parameters of the model (Beaumont & Rannala 2004). This analysis is different from an MLE analysis, in which the outcome is a probability distribution for the data given a fixed set of parameters. Bayesian analyses may be considered to be fully probabilistic because not only are the data treated as a random variable, but the

parameters of the model are also treated as random variables. An appealing feature of Bayesian analysis is that it incorporates previous background information or estimations of known parameters (called "priors") into the model specifications. If detailed estimates of this prior information are not available, partial or incomplete information can be used. A Bayesian analysis will use the probability distribution of the data and the distribution of the priors to calculate the posterior distribution. The posterior distribution can be used to make inferences about the parameters, such as a point estimate of the parameter and its 95% confidence interval. Bayesian analysis can also be used in the analysis of genetic structure of a population, to calculate traditional F_{ST} values, to infer changes in population size, to detect the genetic signatures of selection, and for phylogenetic analysis (Holder & Lewis 2003; Beaumont & Rannala 2004). Recent evolutionary toxicology studies that have used Bayesian analysis include laboratory exposures as well as field studies (Gardeström et al. 2008). Bayesian analyses can also be used to calculate assignment tests (Beaumont & Rannala 2004).

Assignment tests are analyses that use genetic information to establish the likelihood that any particular individual would be found in any given population (Manel et al. 2005). Every individual is assigned to a population in which it has the greatest likelihood of occurrence, given its genotype. Individuals that are incorrectly assigned (i.e., sampled from one population but "assigned" to another) may be immigrants from a different sampled population. This test can also identify individuals that may be from a population other than one that was sampled, if the likelihood of that individual occurring in the sampled population is low. For example, Theodorakis and colleagues (2001, 2006) used assignment tests to examine source–sink dynamics in fish and rodent populations exposed to contaminants. Assignment tests can be based on MLE, Bayesian, or multivariate methods (Manel et al. 2005).

Finally, it should be mentioned that the analyses outlined are not foolproof, and that each type of analysis has its limitations and pitfalls, as well as advantages. Thus, just because differences are found between contaminated and reference populations, it should not automatically be assumed that these differences are due to pollution. Due diligence should be exercised to take into account possible alternative explanations, confounding factors, multiple etiologies, and possible errors or misinterpretations of the results. For example, if MLE, Bayesian analysis, or assignment tests indicate that gene flow occurs between contaminated and reference populations, examination of geographic parameters, dispersal abilities, and life-history traits of the species in question should be used to ensure that such dispersal is likely or even possible. In addition, alternative explanations, such as lack of significant genetic subdivision among populations, a high frequency of shared genotypes among sites, or an asymmetric distribution of private alleles, should also be investigated. Also, most of the analyses rely on specific assumptions – such as mutation–drift or migration–drift equilibrium – that might not be met in contaminated populations. For multiple-loci markers, such as AFLPs or other dominant loci, assumptions of independence among loci may be violated (Luikart et al. 2003). Thus, proper attentiveness to potential pitfalls and limitations of each analysis should be used to avoid inappropriate interpretation or overinterpretation of data.

SUMMARY

Studies in the field of evolutionary toxicology focus on changes to the genetic and evolutionary processes of natural populations that occur from exposure to environmental pollution. Research indicates that integration of population genetics with genetic ecotoxicology provides a useful approach for evaluating postulated long-term and higher-order effects. Detecting and quantifying the influence that environmental pollutants exert on genetic diversity and fitness continue to be the major research challenges facing investigators in this field. Techniques and methodologies of molecular biology are currently being applied to address this challenge. Even though many factors contribute to the success or failure of natural populations, the science of evolutionary toxicology is at a stage of maturity sufficient to begin to delineate the contributions from environmental pollution. Nevertheless, to successfully bridge the gap in our knowledge between exposure to the individual and subsequent population-level consequences, new approaches are needed to help us understand the fundamental biological mechanisms at play.

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